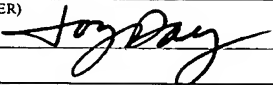


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APPLICATION

for

UNITED STATES LETTERS PATENT

on

**CLONING AND RECOMINANT PRODUCTION OF CRF RECEPTOR(S)**

by

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CLONING AND RECOMBINANT PRODUCTION  
OF CRF RECEPTOR(S)

5

RELATED APPLICATIONS

This application is a continuation-in-part of United States Serial No. 08/483,139, filed June 7, 1995, which is a continuation-in-part of United States Serial  
10 No. 08/353,537, filed December 9, 1994, now pending, which is a continuation-in-part of PCT Application No. PCT/US94/05908, filed May 25, 1994, now pending, which is a continuation-in-part of United States Serial No. 08/110,286, filed August 23, 1993, now pending, which is a  
15 continuation-in-part of United States Serial No. 08/079,320, filed June 18, 1993, now abandoned.

ACKNOWLEDGEMENT

20

This invention was made with United States Government support under Grant Number DK26745, awarded by the National Institutes of Health. The United States Government has certain rights in this invention.

25

FIELD OF THE INVENTION

The present invention relates to receptor proteins, DNA sequences encoding same, and various uses therefor.

### BACKGROUND OF THE INVENTION

Corticotropin-releasing factor (CRF) is a 41-  
5 residue hypothalamic peptide which stimulates the  
secretion and biosynthesis of pituitary  
adrenocorticotrophic hormone (ACTH) leading to increased  
adrenal glucocorticoid production. CRF was originally  
isolated and characterized on the basis of its role in  
10 this hypothalamic-pituitary-adrenal axis (HPA) [Vale et  
al., Science Vol. 213:1394-1397 (1981)]. More recently,  
however, CRF has been found to be distributed broadly  
within the central nervous system (CNS) as well as in  
extra-neural tissues such as the adrenal glands and testes  
15 [Swanson et al., Neuroendocrinology Vol. 36:165-186  
(1983); Suda et al., J. Clin. Endocrinol. Metab. Vol.  
58:919-924 (1984; Fabbri and Dufau, Endocrinology Vol.  
127:1541-1543 (1990)], and sites of inflammation, where it  
may also act as a paracrine regulator or neurotransmitter.

20

In addition to the critical role of CRF in  
mediating HPA axis activation, it has been shown to  
modulate autonomic and behavioral changes that occur  
during the stress response. Many of these behavioral  
25 changes have been shown to occur independently of HPA  
activation in that they are insensitive to dexamethasone  
treatment and hypophysectomy [Britton et al., Life Sci.  
Vol. 38:211-216 (1986); Britton et al., Life Sci. Vol.  
39:1281-1286 (1986); Berridge and Dunn, Pharm. Bioch.  
30 Behav. Vol. 34:517-519 (1989)]. In addition, direct

infusion of CRF into the CNS mimics autonomic and behavioral responses to a variety of stressors [Sutton et al., Nature Vol. 297:331-333 (1982); Brown and Fisher, Brain Res. Vol. 280:75-79 (1983); Stephens et al.,  
5 Peptides Vol. 2:1067-1070 (1988); Butler et al., J. Neurosci. Vol. 10:176-183 (1990)]. Furthermore, peripheral administration of CRF or the CRF antagonist, a-helical CRF 9-41, failed to affect these changes, thus supporting a direct brain action for CRF in such  
10 functions. CRF antagonists given peripherally attenuate stress-mediated increases in ACTH secretion, and when delivered into the cerebral ventricles can mitigate stress induced changes in autonomic activity and behavior.

15 As a result of the extensive anatomical distribution and multiple biological actions of CRF, this regulatory peptide is believed to be involved in the regulation of numerous biological processes. The peptide has been implicated in the regulation of inflammatory  
20 responses. On the one hand, it has been observed that CRF plays a pro-inflammatory role in certain animal models, while in others CRF can suppress inflammation by reducing injury induced increases in vascular permeability.

25 It has also been found that CRF can modify steroid production by the gonads, placenta, and adrenal glands. CRF also has vascular effects such as dilating the superior mesenteric arterial bed and dilating the coronary arteries. In addition to CRF acting on the  
30 central nervous system to modify gastrointestinal

function, CRF has been found to directly effect the gastrointestinal tract as well.

In order to more fully investigate the role of CRF within the endocrine, gastrointestinal, reproductive, central nervous and immune systems, and the possible interactions of CRF with its cognate receptor, it would be desirable to have available a ready source of CRF receptor. Furthermore, the availability of recombinant receptor would allow the development of less expensive, more sensitive, and automated means for assaying CRF and CRF-like compounds and developing CRF-based therapeutics.

The responsivity to CRF or the quantity of CRF receptors in target tissues has been shown or predicted (from altered sensitivity to CRF) to change in response to a variety of circumstances including Alzheimer's Disease, melancholic depression, anorexia nervosa, Cushing's Disease, alcoholism, and the like. Thus, the development of specific anti-CRF-R antibodies and molecular probes for CRF receptor(s) are desired for use in appropriate diagnostic assays.

#### BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided new G-protein-coupled receptor proteins which have high binding affinity for corticotropin-releasing factor (CRF), said proteins are referred to hereinafter as CRF-receptor(s) (CRF-Rs). Invention receptor(s) are principal neuroregulators of the hypothalamic-pituitary-

adrenal cortical axis and play an important role in coordinating the endocrine, autonomic and behavioral responses to stress and immune challenge. CRF-Rs are functionally coupled to adenylate cyclase as it transduces  
5 the signal for CRF-stimulated intracellular cAMP accumulation. Invention CRF-Rs can be employed in a variety of ways, such as, for example, in bioassays, for production of antibodies thereto, in therapeutic compositions containing such proteins and/or antibodies,  
10 and the like.

In accordance with another aspect of the present invention, binding assays employing CRF-Rs are provided, useful for rapidly screening a large number of compounds  
15 to determine which compounds (e.g., agonists and antagonists) are capable of binding to the receptors of the invention. The invention binding assays may also be employed to identify new CRF-like ligands (e.g., putative mammalian sauvagine or urotensin). Test samples (e.g.,  
20 biological fluids) may also be subjected to invention binding assays to detect the presence or absence of CRF or CRF-like compounds.

In accordance with the present invention,  
25 recombinant DNA molecules encoding CRF-Rs are also provided. DNA molecules encoding CRF-Rs (or fragments thereof) are useful, for example, as probes for detecting the presence of CRF-R encoding nucleic acids in biological samples, the identification of additional CRF receptor  
30 proteins, as coding sequences which can be used for the

recombinant expression of the invention receptor proteins (or functional fragments thereof), and the like. Recombinant human CRF-Rs have been expressed in COS cells and bind to CRF and CRF analogs with high affinity. The  
5 recombinant production of CRF-Rs makes feasible their use in the foregoing manners. Fragments of CRF-R encoding nucleic acid can also be employed as primers for PCR amplification of CRF-R encoding DNA. In addition, sequences derived from sequences encoding CRF-Rs can also  
10 be used in gene therapy applications to target the expression of vectors carrying useful genes to specific cell types.

In accordance with another aspect of the present  
15 invention, anti-CRF-R antibodies are also provided. CRF-R and anti-CRF-R antibodies are useful for diagnostic assays to determine levels of CRF-Rs in various tissue samples, e.g., neoplastic tissues, and the like. Anti-CRF-R antibodies can also be used to purify CRF-R protein.  
20 Moreover, these antibodies are considered therapeutically useful to counteract or supplement the biological effect of CRF-Rs in vivo.

Methods and diagnostic systems for determining  
25 the levels of CRF-R in various tissue samples, and levels of CRF-R peptide fragments and CRF in vascular fluid samples, are also provided. These diagnostic methods can be used, for example, for monitoring the level of therapeutically administered CRF-R (or fragments thereof)  
30 to facilitate the maintenance of therapeutically effective

amounts. These diagnostic methods can also be used to diagnose physiological disorders that result from abnormal levels of CRF or CRF-R.

5 CRF-Rs, fragments thereof that bind CRF, or analogs thereof, are capable of therapeutically modulating the effect of CRF. For example, CRF-R fragments can inhibit CRF binding to CRF-R and can inhibit CRF-induced ACTH release in vitro by pituitary cells. Thus, CRF-Rs  
10 can be administered therapeutically in mammals to reduce high ACTH levels caused by excess CRF. Such treatments can be used, for example, to treat Cushing's Disease, and the like. These CRF-Rs are also useful in combating pituitary tumors that produce CRF. Moreover, they can be  
15 used to reduce pituitary ACTH secretion and hence reduce cortisol levels under any condition in which they are abnormally high, such as, for example, during chronic stress, in patients afflicted with anorexia nervosa or alcoholism, and the like. CRF-Rs administered  
20 intravenously (IV) are effective to prevent CRF-induced ACTH release. Furthermore, it is contemplated that IV administration of CRF-Rs can reduce intestinal transit time and thus combat irritable bowel syndrome.

25 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the pharmacologic characteristics of plasmid hctCRFR ("human Cushing's Tumor Corticotropin-releasing factor-receptor"; encoding CRF  
30 receptor subtype hCRF-RA<sub>1</sub>), transiently expressed in COSM6



cells. Figure 1 presents the results of displacement of  $^{125}\text{I}(\text{Nle}^{21}, \text{Tyr}^{32})$  ovine CRF (oCRF) by r/hCRF, when oCRF is bound to membranes prepared from COSM6 cells transfected with hctCRF receptor (n), or rGnRHR ("), as described in Example 3. The data are from one representative experiment repeated at least four times.

Figure 2A illustrates the stimulation of intracellular cAMP in COSM6 cells (transfected with plasmid hctCRF, which encodes CRF receptor subtype CRF-RA<sub>1</sub>) by exposure to CRF, hGRF(1-40)OH, VIP, and Salmon Calcitonin, as described in Example 4.

Figure 2B illustrates the dose-response stimulation of cAMP in COSM6 cells (transfected with plasmid hctCRFR, which encodes CRF receptor subtype CRF-RA<sub>1</sub>) by increasing concentrations of CRF in cells pretreated (n) or untreated (") with the phosphodiesterase inhibitor, IBMX (3-isobutyl-1-methylxanthine).

Figure 2C illustrates the inhibition of CRF stimulated intracellular cAMP by the CRF antagonist  $\alpha$ -helical (9-41) CRF. Each determination is taken from a representative experiment performed in triplicate, repeated at least twice. Cells were pretreated with IBMX. Rat/Human (r/h) CRF was added with (solid bars) or without (hollow bars) 2 mM  $\alpha$ -helical (9-41).

Figures 3A and 3B illustrate a sequence comparison of mouse CRF-RB (SEQ ID NO:10) with mouse CRF-

RA (SEQ ID NO:13). The alignment was made using the Jotun-Hein method with PAM250 residue weight table. Putative transmembrane domains are indicated with a solid bar above the sequence. Potential glycosylation sites are indicated by an (\*).

Figure 4 illustrates results from a competitive displacement of  $^{125}\text{I}$ -(Nle21,Tyr32)-ovine CRF bound to membranes from COSM6 cells transfected with CRF-RB<sub>1</sub>. "T" indicates "total hormone" and "B" indicates "bound hormone." The data are pooled from three independent experiments.

Figure 5 illustrates the accumulation of intracellular cAMP in COSM6 cells transfected with pCRF-RB<sub>1</sub> stimulated by r/hCRF (n), sauvagine (O), suckerfish urotensin (\_), hGRF(1-40) OH (o), and VIP (o). Data are also shown for the inhibition of stimulation when the cells are exposed to 1mM antagonist (DPhe<sup>12</sup>,Nle<sup>21,38</sup>)hCRF(12-41) (t). The data are from one representative experiment described in Example 9, repeated at least twice. The error bars represent the SEM and are smaller than the symbols if not visible.

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a family of isolated mammalian G-protein-coupled CRF-R proteins characterized as having sufficient binding affinity for CRF and CRF-like ligands such that

concentrations of  $\leq 10$  nM of CRF or CRF-like ligands occupy  $\sim 50\%$  of the binding sites of approximately 0.8 nM of said receptor protein (or approximately 10-20 pmol receptor/mg membrane protein).

5

Use of the phrase "isolated" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been produced in such form by the hand of man, and thus are separated from their native in vivo cellular environment. As a result of this human intervention, the recombinant, isolated and/or substantially pure DNAs, RNAs, polypeptides and proteins of the invention can be produced in large quantities and are useful in ways that the DNAs, RNAs, polypeptides or proteins as they naturally occur are not, such as identification of selective drugs or compounds.

20

As used herein, "mammalian" refers to the variety of species from which the invention CRF-R protein is derived, e.g., human, rat, mouse, rabbit, monkey, baboon, bovine, porcine, ovine, canine, feline, and the like. Invention receptors can be derived from a variety of tissue sources, such as, for example, pituitary cells, placental cells, spleen cells, adrenal cells, hematopoietic cells, brain cells, gonadal cells, mesenchymal cells, kidney cells, and the like.

25

As employed herein, the term "CRF-R" refers to a family of isolated and/or substantially pure receptor protein subtypes which participate in the G-protein-coupled response of cells to CRF and CRF-like ligands. Exemplary CRF peptides include r/h CRF and ovine CRF (see U.S. Patent No. 4,415,558), and the like. As employed herein, the phrase "CRF-like ligands" includes substances which have a substantial degree of homology (at least 20% homology) with the amino acid sequence of naturally occurring mammalian CRF, as well as alleles, fragments, homologs or derivatives thereof which have substantially the same biological activity as mammalian CRF. Suitable CRF-like ligands can be obtained from a variety of vertebrate species and include such compounds as sauvagine (see, e.g., U.S. 4,605,642), urotensin (see, e.g., U.S. Patent Nos. 4,908,352; 4,533,654; and 4,525,189) the CRF analogs described in U.S. Patent Nos: 4,415,558; 4,489,163; 4,594,329; 4,605,642; 5,109,111, each of which are incorporated herein by reference, and the like.

20

Such receptor subtypes are typically characterized by having seven putative transmembrane domains, preceded by a large extracellular amino-terminal domain and followed by a large intracellular carboxy-terminal domain. Hydropathy analysis of exemplary invention CRF-Rs (described in SEQ ID NOs: 2, 4, 6 and 10) indicates eight hydrophobic regions of approximately 20 amino acids, corresponding to a possible signal peptide at the N-terminus, plus seven putative transmembrane domains. After removal of the signal peptide, an exemplary

invention receptor (as described, for example, in SEQ ID NO:2) has a molecular weight of approximately 40-45 kilodaltons.

5 Exemplary CRF-R amino acid structures are set forth in SEQ ID NOs 2, 4, 6, 8, 10 and 15 of the Sequence Listing provided hereinafter. The CRF-R described in SEQ ID NO:2 contains five potential glycosylation sites at amino acid positions 38, 45, 78, 90 and 98 (and is referred to herein as CRF-RA<sub>1</sub>). Potential protein kinase C phosphorylation sites are located in the first and second intracellular loops and in the C-terminal tail at positions 146, 222, 386, and 408. Potential casein kinase II and protein kinase A phosphorylation sites are located 15 at positions 301 and 302, respectively. The third intracellular loop of the invention CRF-R set forth in SEQ ID NO:2 contains an amino acid sequence similar to the G<sub>s</sub> activating region found in the third intracellular loop of the b<sub>2</sub>-adrenergic receptor.

20

The invention receptor described in SEQ ID NO:2 exhibits appropriate pharmacologic specificity, i.e., having high affinity for human/rat CRF, ovine CRF, the CRF antagonist a helical (9-41) CRF, (DPhe<sup>12</sup>,Nle<sup>21,38</sup>)hCRF(12- 25 41), urotensins, sauvagine, and very low affinity for the biologically impotent analog, [Ala<sup>14</sup>]-oCRF. A series of non-related peptides are inactive, including such compounds as growth hormone releasing factor, salmon calcitonin, vasoactive intestinal polypeptide, and 30 gonadotropin releasing hormone, as shown in Figure 2C.

Binding affinity (which can be expressed in terms of association constants,  $K_a$ , or dissociation constants,  $K_d$ ) refers to the strength of interaction between ligand and receptor, and can be expressed in terms of the concentration of ligand necessary to occupy one-half (50%) of the binding sites of the receptor. A receptor having a high binding affinity for a given ligand will require the presence of very little ligand to become at least 50% bound (hence the  $K_d$  value will be a small number); conversely, receptor having a low binding affinity for a given ligand will require the presence of high levels of ligand to become 50% bound (hence the  $K_d$  value will be a large number).

15

Reference to receptor protein "having sufficient binding affinity such that concentrations of CRF less or CRF-like peptides than or equal to 10 nM (i.e.,  $\leq 10$  nM) occupy  $\geq 50\%$  (i.e., greater than or equal to one-half) of the binding sites of said receptor protein" means that ligand (i.e., CRF) concentration(s) of no greater than about 10 nM are required in order for the ligand to occupy at least 50% of the active sites of approximately 0.8 nM of said receptor (or approximately 10-20 pmol receptor/mg membrane protein), with much lower ligand concentrations typically being required. Presently preferred receptors are those which have a binding affinity such that ligand concentration(s) in the range of only about 1 - 10 nM are required in order to occupy (or bind to) at least 50% of the receptor binding sites.

30

Members of the invention family of receptors can be divided into various subclasses, based on the degree of similarity between specific members. For example, genomic sequences encoding CRF receptors of the same subclass typically have substantially similar restriction maps, while genomic sequences encoding CRF receptors of different subclasses typically have substantially different restriction maps. In addition, sequences encoding members of the same subclass of receptors will hybridize under high stringency conditions, whereas sequences encoding members of different subclasses will hybridize under low stringency hybridization conditions, but not under high stringency hybridization conditions.

15

Thus, each member of a given subclass is related to other members of the same subclass by having a high degree of homology (e.g., >80% overall amino acid homology) between specific members; whereas members of a given subclass differ from members of a different subclass by having a lower degree of homology (e.g., about 30% up to 80% overall amino acid homology) between specific members of different subclasses.

Based on the above criteria, the receptor species described herein can be designated as CRF-RA or CRF-RB subtypes. Thus, the receptor described in SEQ ID NO:2 is a CRF-RA subtype, and is referred to herein as hCRF-RA<sub>1</sub> (for human CRF-R, subtype A, variant 1). The modified form of hCRF-RA<sub>1</sub> which contains the insert

30

sequence set forth in SEQ ID NO:4 is referred to herein as hCRF-RA<sub>2</sub>. Similarly, the receptor described in SEQ ID NO:6 is referred to herein as rCRF-RA (for rat CRF-R, subtype A), and the receptor described in SEQ ID NOs:8 and 10 are referred to herein as mCRF-RB<sub>1</sub> (for mouse CRF-R, subtype B, variant 1).

The mouse CRF-RA<sub>1</sub> and CRF-RB<sub>1</sub> receptors have been compared and are seen to be about 70% homologous at the nucleotide level and 68% homologous at the amino acid level (see, e.g., Figures 3A and 3B). In addition, there are a number of fundamental structural characteristics that are conserved between the two receptors. The number and location of potential N-glycosylation sites are the same. Six cysteines are present in the N-terminal domain, characteristic of the receptor family. In the CRF-RB<sub>1</sub> receptor, however, there are two additional cysteines, one in the N-terminus and the other at the junction of the first extracellular loop (i.e., ECL-1) and the third transmembrane domain (i.e., TMD-3). If one assumes that the N-terminal cysteine is removed with the signal peptide and that the latter cysteine is within the transmembrane domain, CRF-RB<sub>1</sub> is seen to have six cysteines in the extracellular region, as is the case for other members of the receptor family.

The first intracellular loop is practically the same between CRF-RA<sub>1</sub> and CRF-RB<sub>1</sub>, except for the substitution of a valine for the arginine found in CRF-RA<sub>1</sub>. The second intracellular loop differs in three amino



acids, but the changes are conservative. Thus, a methionine, a glutamic acid and a histidine are present in CRF-RB<sub>1</sub>, instead of a leucine, an aspartic acid and an arginine, respectively, in CRF-RA<sub>1</sub>. The third  
5 intracellular loop is 100% identical between the two receptors. The C-terminal domain is also highly conserved between the two receptors. The putative phosphorylation sites in the intracellular loops are also nearly identical between CRF-RA<sub>1</sub> and CRF-RB<sub>1</sub>, with one exception occurring  
10 in the C-terminus, in which SER386 in CRF-RA<sub>1</sub> is present as an alanine in CRF-RB<sub>1</sub>.

A major determinant of the coupling invention CRF-receptors to GTP-binding proteins and subsequently to  
15 adenylate cyclase is thought to reside in the third intracellular loop and the C-terminus. Because of the high similarity of the third intracellular loop and the C-termini of CRF-RA<sub>1</sub> and CRF-RB<sub>1</sub>, the coupling and signal transduction properties of the two receptors are expected  
20 to be very similar. Indeed, the data (Figure 5) demonstrate that the signal transduction characteristics of CRF-RA<sub>1</sub> and CRF-RB<sub>1</sub> are nearly identical. It is expected that a more detailed analysis of the desensitization characteristics of the two receptors will  
25 reveal subtle differences as a consequence of the changes in the C-terminus and the other intracellular loops.

The main differences between the two receptors are found in the N-terminal domain, in which sixteen extra  
30 amino acids are found in CRF-RB<sub>1</sub>, and in which there are

significant, non-conservative amino acid changes in the remaining portion of the N-terminus. It is interesting to note that, based on the genomic sequence of the mouse CRF-RA<sub>1</sub>, the amino acid sequences of the two receptors  
5 start to diverge very close to the second intron/exon junction in the N-terminus, raising the possibility that some of the divergence between the two receptors could result from alternative exon utilization (i.e., splice variants). Indeed, the presence of multiple protected RNA  
10 species when using N-terminal probes is consistent with the existence of splice variants of this receptor.

In addition to the N-terminal domain, the first, second, and third extracellular loops (ECLs-1, -2, and -3)  
15 also contain significant differences. For example, extracellular loop-1 in CRF-RB<sub>1</sub> contains more charged residues than does the corresponding loop in CRF-RA<sub>1</sub>. Extracellular loop-2 in CRF-RA<sub>1</sub> contains an arginine, instead of a glutamic acid in CRF-RB<sub>1</sub>. Extracellular loop-  
20 3 is the most similar between the two receptors. It is presently believed that a major binding determinant in this family of receptors is the N-terminal region and the extracellular loops. Therefore, the existence of differences in the extracellular domains suggests that the  
25 binding specificities between the two receptors should differ. Urotensin ( $K_i=0.7\pm0.3$ ,  $n=3$ ) and sauvagine ( $K_i=0.6\pm0.1$ ,  $n=3$ ) show a trend to be more potent than r/hCRF ( $K_i=1.3\pm0.2$ ,  $n=6$ ) on CRF-RB.

In situ hybridization studies indicate that mRNAs related to CRF-RB have a restricted distribution in the central nervous system that differs considerably from that of CRF-RA. Thus, the receptors derived from the two  
5 genes, CRF-RA and CRF-RB, with their distinct tissue distributions and structural diversity especially in the extracellular domains are likely to subserve disparate biological roles.

10 It is expected that a more detailed pharmacological comparison of the two receptors is likely to reveal significant differences in the binding characteristics of the two receptors. It is also expected that different CRF-R subtypes will mediate different  
15 actions of CRF. Thus, by having available nucleic acid encoding various CRF-R subtypes, those of skill in the art have been enabled to screen for and develop selective analogs specific for each CRF-R subtype. The analogs so obtained will be more specific, potent, and effective at  
20 binding and modulating the activity of the respective CRF-R subtype.

In one embodiment of the present invention, the CRF-RA<sub>1</sub> encoded by the clone referred to herein as  
25 "hctCRFR" (described hereinafter) has a high binding affinity for r/h CRF [ $K_d=3.3\pm0.45$  nM (n=4)]; ovine CRF [ $K_d=2.3\pm0.66$  nM (n=3)]; and for the antagonist a helCRF(9-41) [ $K_d=13.0\pm5.2$  nM (n=3)]. This receptor has a low binding affinity for the biologically impotent analog,  
30 [Ala<sup>14</sup>]-ovine CRF [ $K_d>300$  nM (n=2)]. In another embodiment

of the present invention, the CRF-R described in SEQ ID NO:2 has a binding affinity for r/h CRF of  $K_d=3.8\pm0.20$  nM, (n=1).

5                   Presently preferred receptor proteins of the invention have amino acid sequences that are substantially the same as the sequences set forth in Sequence ID Nos. 2, 4, 6, 8, and 10 and amino acid sequences which are substantially the same as the amino acid sequences encoded  
10 by the CRF-RA<sub>1</sub>-encoding portion of clone htcCRFR, deposited with the ATCC under accession number 75474, as well as functional, modified forms thereof. Those of skill in the art recognize that numerous residues of the above-described sequences can be substituted with other,  
15 chemically, sterically and/or electronically similar residues without substantially altering the biological activity of the resulting receptor species.

                  The htcCRFR clone was deposited June 2, 1993, at  
20 the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20852, under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under  
25 this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States  
30 of America and all other nations or international

organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted. In particular, upon issuance of a U.S. patent  
5 based on this or any application claiming priority to or incorporating this application by reference thereto, all restriction upon availability of the deposited material will be irrevocably removed.

10 As employed herein, the term "substantially the same amino acid sequence" refers to amino acid sequences having at least about 70% identity with respect to the reference amino acid sequence, and retaining comparable functional and biological properties characteristic of the  
15 protein defined by the reference amino acid sequence. Preferably, proteins having "substantially the same amino acid sequence" will have at least about 80%, more preferably 90% amino acid identity with respect to the reference amino acid sequence; with greater than about 95%  
20 amino acid sequence identity being especially preferred.

Recombinant CRF-R protein can be routinely obtained, employing the invention nucleic acids described hereinafter, having significantly higher purity than  
25 naturally occurring CRF-R (e.g., substantially free of other proteins present in crude extracts from mammalian cells). Recombinant DNA techniques well-known in the art, for example, can be used to generate organisms or cell lines that produce heterologous CRF-R protein in  
30 significantly higher purities, relative to naturally

occurring membrane protein. Subsequently, using appropriate isolation techniques, it is possible to routinely obtain CRF-R proteins which are at least about 70%, preferably 80%, more preferably 90%, and most preferred 98% pure (by weight of total proteins), and which is herein referred to as substantially pure.

In accordance with a further embodiment of the present invention, there is provided a binding assay employing receptors of the invention, whereby a large number of compounds can be rapidly screened to determine which compounds, if any, are capable of binding to the receptors of the invention. Subsequently, more detailed assays can be carried out with initially identified compounds, to further determine whether such compounds act as agonists or antagonists of invention receptors.

Another application of the binding assay of the invention is the assay of test samples (e.g., biological fluids) for the presence or absence of CRF. Thus, for example, serum from a patient displaying symptoms thought to be related to over- or under-production of CRF can be assayed to determine if the observed symptoms are indeed caused by over- or under-production of CRF (or CRF receptor).

The binding assays contemplated by the present invention can be carried out in a variety of ways, as can readily be identified by one of skill in the art. For

example, competitive binding assays can be employed, as well as radioimmunoassays, ELISA, ERMA, and the like.

In accordance with a still further embodiment of the present invention, there are provided bioassays for evaluating whether test compounds are capable of acting as agonists or antagonists of receptor(s) of the present invention (or functional modified forms thereof).

Invention CRF-Rs are coupled by heterotrimeric G-proteins to various intracellular enzymes, ion channels, and transporters. The G-proteins associate with invention CRF-R proteins at the intracellular face of the plasma membrane. An agonist binding to CRF-R catalyzes the exchanges of GTP for GDP on the  $\alpha$ -subunit (G-protein "activation"), resulting in its dissociation and stimulation of one (or more) of the various signal-transducing enzymes and channels. The different G-protein  $\alpha$ -subunits preferentially stimulate particular effectors. The specificity of signal transduction may be determined, therefore, by the specificity of G-protein coupling.

It has been found that invention CRF-R proteins mediate signal transduction through the modulation of adenylate cyclase. For example, when CRF binds to CRF-R, adenylate cyclase causes an elevation in the level of intracellular cAMP. Accordingly, in one embodiment of the present invention, the bioassay for evaluating whether test compounds are capable of acting as agonists or antagonists comprises:

(a) culturing cells containing:

DNA which expresses CRF receptor  
protein(s) or functional modified  
forms thereof,

5 wherein said culturing is carried out in  
the presence of at least one compound whose  
ability to modulate signal transduction  
activity of CRF receptor protein is sought  
to be determined, and thereafter

10 (b) monitoring said cells for either an  
increase or decrease in the level of  
intracellular cAMP.

Methods well-known in the art that measure  
15 intracellular levels of cAMP, or measure cyclase activity,  
can be employed in binding assays described herein to  
identify agonists and antagonists of the CRF-R. For  
example, because activation of some G-protein-coupled  
receptors results in decreases or increases in cAMP,  
20 assays that measure intracellular cAMP levels (see, e.g.,  
Example 4) can be used to evaluate recombinant CRF-Rs  
expressed in mammalian host cells.

As used herein, "ability to modulate signal  
25 transduction activity of CRF receptor protein" refers to a  
compound that has the ability to either induce or inhibit  
signal transduction activity of the CRF receptor protein.



In another embodiment of the present invention, the bioassay for evaluating whether test compounds are capable of acting as agonists comprises:

(a) culturing cells containing:

5 DNA which expresses CRF receptor protein(s) or functional modified forms thereof, and

DNA encoding a reporter protein, wherein said DNA is operatively linked to a CRF-R responsive transcription element;

10 wherein said culturing is carried out in the presence of at least one compound whose ability to induce signal transduction activity of CRF receptor protein is sought to be determined, and thereafter

15 (b) monitoring said cells for expression of said reporter protein.

20 In another embodiment of the present invention, the bioassay for evaluating whether test compounds are capable of acting as antagonists for receptor(s) of the invention, or functional modified forms of said receptor(s), comprises:

25 (a) culturing cells containing:

DNA which expresses CRF receptor protein(s), or functional modified forms thereof, and

30 DNA encoding a reporter protein, wherein said DNA is operatively linked

to a CRF-R responsive transcription  
element

wherein said culturing is carried out in  
the presence of:

5                    increasing concentrations of at  
least one compound whose ability to  
inhibit signal transduction activity  
of CRF receptor protein(s) is sought  
to be determined, and

10                   a fixed concentration of at least  
one agonist for CRF receptor  
protein(s), or functional modified  
forms thereof; and thereafter

(b) monitoring in said cells the level of  
15                   expression of said reporter protein as a  
function of the concentration of said  
compound, thereby indicating the ability of  
said compound to inhibit signal  
transduction activity.

20

In step (a) of the above-described antagonist  
bioassay, culturing may also be carried out in the  
presence of:

25                   fixed concentrations of at least  
one compound whose ability to inhibit  
signal transduction activity of CRF  
receptor protein(s) is sought to be  
determined, and

30                   an increasing concentration of at  
least one agonist for CRF receptor

protein(s), or functional modified forms thereof.

Host cells for functional recombinant expression  
5 of CRF-Rs preferably express endogenous or recombinant  
guanine nucleotide-binding proteins (i.e., G-proteins).  
G-proteins are a highly conserved family of membrane-  
associated proteins composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. The  
 $\alpha$  subunit, which binds GDP and GTP, differs in different  
10 G-proteins. The attached pair of  $\beta$  and  $\gamma$  subunits may or  
may not be unique; different  $\alpha$  chains may be linked to an  
identical  $\beta\gamma$  pair or to different pairs [Linder and  
Gilman, *Sci. Am.* 267:56-65 (1992)]. More than 30  
different cDNAs encoding G protein  $\alpha$  subunits have been  
15 cloned [Simon et al., *Science* 252:802 (1991)]. At least  
four different  $\beta$  polypeptide sequences are known [Simon et  
al., *Science* 252:802 (1991)]. G-proteins switch between  
active and inactive states by guanine nucleotide exchange  
and GTP hydrolysis. Inactive G protein is stimulated by a  
20 ligand-activated receptor to exchange GDP for GTP. In the  
active form, the  $\alpha$  subunit, bound to GTP, dissociates from  
the  $\beta\gamma$  complex, and the subunits then interact  
specifically with cellular effector molecules to evoke a  
cellular response. Because different G-proteins can  
25 interact with different effector systems (e.g.,  
phospholipase C, adenylyl cyclase systems) and different  
receptors, it is useful to investigate different host  
cells for expression of different recombinant CRF-R  
receptor subtypes. Alternatively, host cells can be

transfected with G-protein subunit-encoding DNAs for heterologous expression of differing G proteins.

Host cells contemplated for use in the  
5 bioassay(s) of the present invention include CV-1 cells, COS cells, and the like; reporter and expression plasmids employed typically also contain the origin of replication of SV-40; and the reporter and expression plasmids employed also typically contain a selectable marker.

10

As used herein, a "CRF-R responsive transcription element" is any promoter region that is induced, e.g., by the well-known G-protein mediated signal transduction mechanism, to initiate transcription upon the  
15 binding of a CRF-R agonist, such as CRF. A preferred CRF-R responsive transcription element is a cAMP responsive transcription element. Cyclic AMP (cAMP) responsive transcription elements employed in the bioassay(s) of the present invention are well-known to those of skill in the  
20 art. The cAMP response elements respond to increases in intracellular cAMP by initiating transcription of the DNA molecule (i.e., a reporter gene) operatively linked thereto. An exemplary cAMP response element suitable for use herein is the human DNA b-Polymerase gene promoter  
25 (see Mamula et al., DNA and Cell Bio., 11:61-70, 1992).

Reporter proteins suitable for use herein are well known in the art. Host cells can be monitored for the level of expression of a reporter gene encoding a  
30 reporter protein in a variety of ways, such as, for

example, by photometric means, e.g., by colorimetry (with a colored reporter product such as  $\beta$ -galactosidase), by fluorescence (with a reporter product such as luciferase), by enzyme activity, and the like.

5

Compounds contemplated for screening in accordance with the invention bioassays include CRF or CRF-like ligands, as well as compounds which bear no particular structural or biological relatedness to CRF.

10 Suitable compounds may be obtained from well-known sources, e.g., from peptide libraries, chemical libraries, bacterial and yeast broths, plants, and the like.

Examples of compounds which bear no particular structural or biological relatedness to CRF, but which are contemplated for screening in accordance with the bioassays of the present invention, include any compound that is an antagonist (i.e., is capable of blocking the action of the invention receptor peptides), or an agonist

15 (i.e., is capable of promoting the action of the invention receptor peptides), such as, for example, alkaloids and other heterocyclic organic compounds, and the like.

20

As employed herein, the term "non-CRF-like" proteins refers to any organic molecule having essentially no structural similarity with CRF (as defined broadly herein).

25

Also encompassed by the term CRF-R are the various subtypes thereof (e.g., CRF-RA (such as hCRF-RA<sub>1</sub>

30

and hCRF-RA<sub>2</sub>), CRF-RB<sub>1</sub>, and the like), as well as polypeptide fragments or analogs thereof. Therefore, a CRF-R contemplated by the present invention can be subject to various changes, substitutions, insertions, and deletions, where such changes provide for certain advantages in its use. For example, a peptide fragment is capable of immunologically mimicking a CRF-R native antigenic epitope or is capable of exhibiting another biological property characteristic of CRF-R, such as, for example, binding to CRF or binding to G-protein(s).

Specific CRF-R residues or regions which are necessary for efficient signal transduction may interact with conserved G-protein motifs. In addition, certain short amino acid stretches of the CRF-R, which are necessary for G-protein coupling, also determine the specificity of the G-protein interactions. Thus, polypeptide fragments of the invention CRF-R are useful in assays or therapeutic methods in which controlled binding to various G-proteins is desired.

The term "analog" includes any polypeptide having an amino acid residue sequence substantially identical to a sequence specifically shown herein in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to mimic CRF-R as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the

substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine  
5 or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also  
10 includes the use of a chemically derivatized residue in place of a non-derivatized residue, provided that such polypeptide displays the requisite binding activity.

"Chemical derivative" refers to a subject  
15 polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl  
20 groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine  
25 may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For  
30 examples: 4-hydroxyproline may be substituted for

proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions of residues, relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

When additional residues have been added at either terminus for the purpose of providing a "linker" by which the polypeptides of the invention can be conveniently affixed to a label or solid matrix, or carrier, the linker residues do not form CRF-R epitopes, i.e., are not similar in structure to CRF-R. Labels, solid matrices, and carriers that can be used with the polypeptides of this invention are described hereinbelow.

Amino acid residue linkers include at least one residue up to 40 or more residues (more often they comprise 1 to 10 residues), but do not form CRF-R epitopes. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic acid and aspartic acid. In addition, a subject polypeptide can differ in sequence, unless otherwise specified, from the natural sequence of CRF-R by modification of the sequence by N-terminal acylation e.g., acetylation or thioglycolic acid amidation, and by C-terminal amidation, e.g., with ammonia, methylamine, and the like.



CRF-R polypeptides of the present invention are capable of inducing antibodies that immunoreact with CRF-R. In view of the well established principle of immunologic cross-reactivity, the present invention  
5 therefore contemplates antigenically related variants of the polypeptides. An "antigenically related variant" is a subject polypeptide that is capable of inducing antibody molecules that immunoreact with the CRF-R polypeptides described herein.

10

CRF-R polypeptides of the present invention can be synthesized by any of the techniques that are known to those skilled in the polypeptide art, including recombinant DNA techniques. Synthetic chemistry  
15 techniques, such as solid-phase Merrifield-type synthesis, are preferred for producing polypeptide fragments for reasons of purity, antigenic specificity, freedom from undesired side products, ease of production, and the like. An excellent summary of the many techniques available can  
20 be found in J.M. Steward and J.D. Young, "Solid Phase Peptide Synthesis", W.H. Freeman Co., San Francisco, 1969; M. Bodansky, et al., "Peptide Synthesis", John Wiley & Sons, Second Edition, 1976 and J. Meienhofer, "Hormonal Proteins and Peptides", Vol. 2, p. 46, Academic Press (New  
25 York), 1983 for solid phase peptide synthesis, and E. Schroder and K. Kubke, "The Peptides", Vol. 1, Academic Press (New York), 1965 for classical solution synthesis, each of which is incorporated herein by reference. Appropriate protective groups usable in such synthesis are  
30 described in the above texts and in J.F.W. McOmie,

"Protective Groups in Organic Chemistry", Plenum Press, New York, 1973, which is incorporated herein by reference. See also U.S. Patent No. 5,055,396, incorporated herein by reference.

5

CRF-R polypeptides can be used, inter alia, in diagnostic methods and systems according to the present invention to detect the level of CRF-R (or fragments thereof) present in a body sample, to detect the level of  
10 CRF in a body sample, or to prepare an inoculum as described herein for the preparation of antibodies that immunoreact with epitopes on CRF-R. CRF-R polypeptides can also be used to bind, detect and purify various intracellular G-proteins and CRF-like receptor  
15 agonist/antagonists, such as heterocyclic compounds, and the like. In addition, CRF-R polypeptides can be used in therapeutic methods described herein, e.g., to inhibit the CRF-induced ACTH release and decrease the level of ACTH in a patient.

20

In accordance with yet another embodiment of the present invention, there are provided antibodies generated against the above-described receptor proteins. Such antibodies can be employed for diagnostic applications,  
25 therapeutic applications, and the like. Preferably, for therapeutic applications, the antibodies employed will be monoclonal antibodies.

The above-described antibodies can be prepared  
30 employing standard techniques, as are well known to those

of skill in the art, using invention receptor proteins, or fragments thereof, as antigens for antibody production. Antibodies of the present invention are typically produced by immunizing a mammal with an inoculum containing a CRF-R  
5 protein or fragment thereof thereby inducing the production of antibody molecules having immunospecificity for the immunizing agent.

For example, antibodies raised in rabbits  
10 against a synthetic peptide fragment of the invention protein recognize the synthetic peptide and the corresponding invention CRF-R on an equimolar basis, and preferably, are capable of inhibiting the activity of the native protein. Antibodies to CRF-R may be obtained, for  
15 example, by immunizing three month old male and female white New Zealand rabbits with a suitable synthetic peptide fragment to which Tyr has been added at the C-terminus in order to couple it, as an antigen, to BSA by a bisdiazotized benzidine (BDB) linkage by reaction for 2  
20 hours at 4°C. The reaction mixture is dialyzed to remove low molecular weight material, and the retentate is frozen in liquid nitrogen and stored at -20°C. Animals are immunized with the equivalent of 1 mg of the peptide antigen according to the procedure of Vaughan et al.,  
25 Meth. in Enzymology, 168:588-617 (1989). At four week intervals, the animals are boosted by injections of 200 mg of the antigen and bled ten to fourteen days later. After the third boost, antiserum is examined for its capacity to bind radioiodinated antigen peptide prepared by the  
30 chloramine-T method and then purified by CMC-ion exchange

column chromatography or HPLC. The antibody molecules are then collected from the mammal and isolated to the extent desired by well known techniques such as, for example, by using DEAE Sephadex to obtain the IgG fraction.

5

To enhance the specificity of the antibody, the antibodies may be purified by immunoaffinity chromatography using solid-phase immunizing polypeptide. The antibody is contacted with the solid-phase immunizing polypeptide for a period of time sufficient for the polypeptide to immunoreact with the antibody molecules to form a solid-phase immunocomplex. The bound antibodies are separated from the complex by standard techniques.

15

Antibody so produced can be used, inter alia, in diagnostic methods and systems to detect the level of CRF-R present in a mammalian, preferably human, body sample, such as tissue or vascular fluid. The anti-CRF-R antibodies can also be used for immunoaffinity or affinity chromatography purification of CRF-R biological materials. In addition, an anti-CRF-R antibody according to the present invention can be used in mammalian therapeutic methods, preferably human, as a CRF-R agonist or antagonist, to neutralize or modulate the effect of CRF-R, increase the level of free CRF (e.g., CRF not bound by CRF-R), increase CRF-induced ACTH release, increase the level of ACTH-induced glucocorticoids in a patient, and the like.

20  
25

The proteins of the invention, and the antibodies of the invention, can be administered to a subject employing standard methods, such as, for example, by intraperitoneal, intramuscular, intravenous, or subcutaneous injection, and the like. Implant and transdermal modes of administration are also appropriate. In addition, proteins of the invention can be delivered by transfection with viral or retroviral vectors that encode invention protein. One of skill in the art can readily determine dose forms, treatment regimens, etc, depending on the mode of administration employed.

In accordance with a further embodiment of the present invention, there are provided isolated and purified nucleic acid molecules (e.g., DNA or RNA) which encode the above-described receptor proteins. The nucleic acid molecules described herein are useful for producing invention CRF-R proteins, when such nucleic acids are incorporated into a variety of protein expression systems known to those of skill in the art. In addition, such nucleic acid molecules (or fragments thereof) can be labeled with a readily detectable substituent and used as hybridization probes for assaying for the presence and/or amount of a CRF-R gene or mRNA transcript in a given sample. Such nucleic acid molecules (or fragments thereof), when labeled with a readily detectable substituent, can also be used as hybridization probes for identifying additional CRF-R genes. The nucleic acid molecules described herein, and fragments thereof, are also useful as primers and/or templates in a PCR reaction

for amplifying genes encoding the CRF-R protein described herein. In addition, the nucleic acid molecules described herein, and fragments thereof, are also useful as primers and/or templates in a PCR reaction for identifying genes  
5 encoding additional CRF-R proteins which are part of the family of receptor proteins described herein.

The above-described receptor(s) can be encoded by numerous nucleic acid molecules, e.g., a nucleic acid  
10 molecule having a contiguous nucleotide sequence substantially the same as:

nucleotides 82 - 1329 of Sequence ID No. 1,  
nucleotides 82 - 1329 of Sequence ID No. 1,  
further containing nucleotides 1-87 of SEQ ID  
15 No. 3 inserted between nucleotides 516-517 of SEQ ID No. 1,

nucleotides 81-1324 of Sequence ID No. 5,  
substantially all nucleotides of Sequence  
ID No. 7,

20 nucleotides 79-1371 of Sequence ID No. 9,  
the CRF-RA<sub>1</sub>-encoding portion of clone hctCRFR, deposited with the ATCC under accession number 75474,

or variations thereof which encode the same  
25 amino acid sequences, but employ different codons for some of the amino acids,

or splice variant cDNA sequences thereof.

As employed herein, the phrase "nucleic acid"  
30 refers to ribonucleic acid (RNA) or deoxyribonucleic acid

(DNA). DNA can be either complementary DNA (cDNA) or genomic DNA, e.g. a gene encoding a CRF-R.

As employed herein, the phrases "contiguous  
5 nucleotide sequence substantially the same as" or  
"substantially the same nucleotide sequence" refers to DNA  
having sufficient homology to the reference  
polynucleotide, such that it will hybridize to the  
reference nucleotide under typical moderate stringency  
10 conditions. In one embodiment, nucleic acid molecules  
having substantially the same nucleotide sequence as the  
reference nucleotide sequence encodes substantially the  
same amino acid sequence as that of any one of SEQ ID  
NOs:2, 4, 6, 8, or 10. In another embodiment, DNA having  
15 "substantially the same nucleotide sequence" as the  
reference nucleotide sequence has at least 60% homology  
with respect to the reference nucleotide sequence. DNA  
having at least 70%, more preferably 80%, yet more  
preferably 90%, homology to the reference nucleotide  
20 sequence is preferred.

Yet other DNAs which encode the above-described  
receptor are those having a contiguous nucleotide sequence  
substantially the same as set forth in Sequence ID Nos. 1,  
25 3, 5, 7, or 9 or the CRF-RA<sub>1</sub>-encoding portion of clone  
hctCRFR, deposited with the ATCC under accession number  
75474.

"Gene(s)" (i.e., genomic DNA) encoding invention  
30 CRF-Rs typically contain at least two introns. Thus,

alternatively spliced variant cDNA sequences encoding invention CRF-Rs are contemplated herein (e.g., CRF-RA<sub>2</sub>). For example, SEQ ID NO:3 sets forth an 87 bp cDNA splice variant insert sequence that is inserted between  
5 nucleotide positions 516-517 of the CRF-RA<sub>1</sub> encoding cDNA set forth in SEQ ID NO:1 (thereby producing CRF-RA<sub>2</sub>).

As used herein, the phrases "splice variant" or "alternatively spliced", when used to describe a  
10 particular nucleotide sequence encoding an invention receptor, refers to a cDNA sequence that results from the well known eukaryotic RNA splicing process. The RNA splicing process involves the removal of introns and the joining of exons from eukaryotic primary RNA transcripts  
15 to create mature RNA molecules of the cytoplasm.

Methods of isolating splice variant nucleotide sequences are well known in the art. For example, one of skill in the art can employ nucleotide probes derived from  
20 the CRF-R encoding cDNA of SEQ ID NOS 1, 3, 5, 7, or 9 to screen the Cushing's tumor cDNA library described in the Examples or other cDNA libraries derived from cells believed to express CRF-Rs, e.g., brain, heart, pituitary, immune, gonadal, adrenal, placental, gastrointestinal,  
25 pulmonary, corticotropic, and the like.

In a preferred embodiment, cDNA encoding CRF-Rs as disclosed herein have substantially the same nucleotide sequence as nucleotides 82-1329 of SEQ ID NO:1, as  
30 nucleotides 82-1329 of SEQ ID NO:1 further containing



nucleotides 1-87 of SEQ ID NO:3 inserted between  
nucleotides 516-517 of SEQ ID NO:1, as SEQ ID NO:5, as  
nucleotides 81-1324 of SEQ ID NO:7, or as nucleotides 79-  
1371 of SEQ ID NO:9. The presently most preferred cDNA  
5 molecules encoding the CRF-Rs have the same nucleotide  
sequence as nucleotides 82-1329 of SEQ ID NO:1, as  
nucleotides 82-1329 of SEQ ID NO:1 further containing  
nucleotides 1-87 of SEQ ID NO:3 inserted between  
nucleotides 516-517 of SEQ ID NO:1, as SEQ ID NO:5, as  
10 nucleotides 81-1324 of SEQ ID NO:7, or as nucleotides 79-  
1371 of SEQ ID NO:9.

In accordance with another embodiment of the  
present invention, isolated and purified nucleic acid  
15 encoding a CRF-R may be selected from:

(a) DNA encoding the amino acid sequence  
set forth in SEQ ID NO:2, SEQ ID NO:6, SEQ ID  
NO:8 or SEQ ID NO:10; or DNA encoding the amino  
acid sequence set forth in SEQ ID NO:2 further  
20 comprising the amino acid sequence set forth in  
SEQ ID NO:4 inserted between amino acids 145-146  
of SEQ ID NO:2, or

(b) DNA that hybridizes to the DNA of (a)  
under moderately stringent conditions, wherein  
said DNA encodes biologically active CRF-R, or  
25

(c) DNA degenerate with respect to either  
(a) or (b) above, wherein said DNA encodes  
biologically active CRF-R.

Hybridization refers to the binding of complementary strands of nucleic acid (i.e., sense:antisense strands or probe:target-DNA) to each other through hydrogen bonds, similar to the bonds that naturally occur in chromosomal DNA. Stringency levels used to hybridize a given probe with target-DNA can be readily varied by those of skill in the art.

As used herein, the phrase "moderately stringent" hybridization refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60%, preferably about 75%, more preferably about 85%, homology to the target DNA; with greater than about 90% homology to target-DNA being especially preferred. Preferably, moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C. Denhart's solution and SSPE (see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers.

The term "functional" or "biologically active", when used herein as a modifier of receptor protein(s) of the present invention, refers to a polypeptide that is able to produce one of the functional characteristics, e.g., antigenicity, exhibited by any of the CRF-Rs described herein. In another embodiment, biologically active means that binding of CRF-like ligands (such as CRF

analogs, urotensin, sauvagine, and the like) to said receptor protein(s) modifies the receptor interaction with G-proteins, which in turn affects the levels of intracellular second messengers, preferably cAMP, leading to a variety of physiological effects. Stated another way, "functional" means that a signal is transduced as a consequence of agonist activation of receptor protein(s).

As used herein, the term "degenerate" refers to codons that differ in at least one nucleotide from a reference nucleic acid, e.g., SEQ ID NO:1, but encode the same amino acids as the reference nucleic acid. For example, codons specified by the triplets "UCU", "UCC", "UCA", and "UCG" are degenerate with respect to each other since all four of these codons encode the amino acid serine.

The invention nucleic acids can be produced by a variety of methods well-known in the art, e.g., the methods described in Examples 1 and 5, employing PCR amplification using oligonucleotide primers from various regions of SEQ ID NOs:1, 3, 5, 7, 9 and 14 and the like.

One method employed for isolating and cloning nucleic acids encoding the receptor(s) of the present invention involves expressing, in mammalian cells, a cDNA library prepared from any cell type thought to respond to CRF (e.g., pituitary cells, placental cells, fibroblast cells, and the like) in a suitable host cell, such as, for example, COSM6 cells. The ability of the resulting

mammalian cells to bind a labeled receptor ligand (i.e., a labeled CRF analog) is then determined. Finally, the desired cDNA insert(s) are recovered, based on the ability of a particular cDNA, when expressed in mammalian cells, to induce (or enhance) the binding of labeled receptor ligand to said cell.

Alternatively, DNA libraries may be screened employing an immunological expression assay with an antibody raised against the protein of interest. Screening of the expression library with antibodies raised against the protein of interest may also be used, alone or in conjunction with hybridization probing, to identify or confirm the presence of the sought-after DNA sequences in DNA library clones. Such techniques are taught, for example, in Maniatis et al., Cold Spring Harbor Laboratory Manual, Cold Spring Harbor, New York (1982), (hereinafter CSH).

In accordance with a further embodiment of the present invention, optionally labeled receptor-encoding cDNAs, or fragments thereof, can be employed to probe library(ies) (e.g., cDNA, genomic, and the like) for additional nucleotide sequences encoding novel mammalian members of the CRF receptor family. Such screening is initially carried out under low-stringency conditions, which comprise a temperature of less than about 42.5°C, a formamide concentration of less than about 50%, and a moderate to low salt concentration. Presently preferred screening conditions comprise a temperature of about

42.5°C, a formamide concentration of about 20%, and a salt concentration of about 5X standard saline citrate (SSC; 20X SSC contains 3M sodium chloride, 0.3M sodium citrate, pH 7.5). Such conditions will allow the identification of sequences which have a substantial degree of similarity with the probe sequence, without requiring perfect homology for the identification of a stable hybrid. The phrase "substantial similarity" refers to sequences which share at least 50% homology. Preferably, hybridization conditions will be selected which allow the identification of sequences having at least 70% homology with the probe, while discriminating against sequences which have a lower degree of homology with the probe.

As used herein, a nucleic acid "probe" is single-stranded DNA or RNA, or analogs thereof, that has a sequence of nucleotides that includes at least 14, preferably at least 20, more preferably at least 50, contiguous bases that are the same as (or the complement of) any 14 or more contiguous bases set forth in any of SEQ ID NOs: 1, 3, 5, 7 or 9 or the CRF-RA<sub>1</sub>-encoding portion of clone hctCRFR. Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode cytoplasmic loops, signal sequences, ligand binding sites, and the like. The entire cDNA molecule encoding an invention CRF-R may also be employed as a probe. Probes may be labeled by methods well-known in the art, as described hereinafter, and used in various diagnostic kits.

In accordance with yet another embodiment of the present invention, there is provided a method for the recombinant production of invention receptor(s) by  
5 expressing the above-described nucleic acid sequences in suitable host cells. The above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce  
10 heterologous DNA (e.g., SEQ ID NOS:1, 3, 5, 7 or 9) into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan.

15 An expression vector includes elements capable of expressing DNAs that are operatively linked with regulatory sequences (such as promoter regions) that are capable of regulating expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or  
20 RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are  
25 replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently preferred plasmids for expression of invention CRF-Rs in eukaryotic host cells, particularly mammalian cells, include  
30 cytomegalovirus (CMV) promoter-containing vectors, SV40

promoter-containing vectors, MMTV LTR promoter-containing vectors, and the like.

As used herein, a promoter region refers to a  
5 segment of DNA that controls transcription of DNA to which  
it is operatively linked. The promoter region includes  
specific sequences that are sufficient for RNA polymerase  
recognition, binding and transcription initiation. This  
portion of the promoter region is referred to as the  
10 promoter. In addition, the promoter region includes  
sequences that modulate this recognition, binding and  
transcription initiation activity of RNA polymerase.  
These sequences may be cis acting or may be responsive to  
trans acting factors. Promoters, depending upon the  
15 nature of the regulation, may be constitutive or  
regulated. Exemplary promoters contemplated for use in  
the practice of the present invention include the SV40  
early promoter, the cytomegalovirus (CMV) promoter, the  
mouse mammary tumor virus (MMTV) steroid-inducible  
20 promoter, Moloney murine leukemia virus (MMLV) promoter,  
and the like.

As used herein, the term "operatively linked"  
refers to the functional relationship of DNA with  
25 regulatory and effector sequences of nucleotides, such as  
promoters, enhancers, transcriptional and translational  
stop sites, and other signal sequences. For example,  
operative linkage of DNA to a promoter refers to the  
physical and functional relationship between the DNA and  
30 the promoter such that the transcription of such DNA is

initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter  
5 5' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively,  
10 consensus ribosome binding sites (see, for example, Kozak (1991) J. Biol. Chem. 266:19867-19870) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

15

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA,  
20 expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Prokaryotic transformation vectors are well-known in the art and include pBlueskript and phage Lambda  
25 ZAP vectors (Stratagene, La Jolla, CA), and the like. Other suitable vectors for transformation of E. coli cells include the pET expression vectors (Novagen, see U.S patent 4,952,496), e.g., pET11a, which contains the T7 promoter, T7 terminator, the inducible E. coli lac  
30 operator, and the lac repressor gene; and pET 12a-c, which



contain the T7 promoter, T7 terminator, and the E. coli ompT secretion signal. Another suitable vector is the pIN-IIIompA2 (see Duffaud et al., Meth. in Enzymology, 153:492-507, 1987), which contains the lpp promoter, the  
5 lacUV5 promoter operator, the ompA secretion signal, and the lac repressor gene.

Particularly preferred base vectors for transfection of mammalian cells are cytomegalovirus (CMV)  
10 promoter-based vectors such as pcDNA1 (Invitrogen, San Diego, CA), MMTV promoter-based vectors such as pMAMNeo (Clontech, Palo Alto, CA) and pMSG (Catalog No. 27-4506-01 from Pharmacia, Piscataway, NJ), and SV40 promoter-based  
vectors such as pSVb (Clontech, Palo Alto, CA), and the  
15 like.

The use of a wide variety of organisms has been described for the recombinant production of proteins or biologically active fragments thereof. One of skill in  
20 the art can readily determine suitable hosts (and expression conditions) for use in the recombinant production of the peptides of the present invention. Yeast hosts, bacterial hosts, mammalian hosts, and the like can be employed.

25

In accordance with another embodiment of the present invention, there are provided "recombinant cells" containing the nucleic acid molecules (i.e., DNA or mRNA) of the present invention (e.g., SEQ ID NOS:1, 3, 5, 7 or  
30 9). Methods of transforming suitable host cells, as well

as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1989).

Exemplary methods of transformation include, e.g., transformation employing plasmids, viral, or bacterial phage vectors, transfection, electroporation, lipofection, and the like. The heterologous nucleic acid can optionally include sequences which allow for its extrachromosomal maintenance, or said heterologous nucleic acid can be caused to integrate into the genome of the host (as an alternative means to ensure stable maintenance in the host).

Host organisms contemplated for use in the practice of the present invention include those organisms in which recombinant production of heterologous proteins has been carried out. Examples of such host organisms include bacteria (e.g., *E. coli*), yeast (e.g., *Saccharomyces cerevisiae*, *Candida tropicalis*, *Hansenula polymorpha* and *P. pastoris*; see, e.g., U.S. Patent Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), mammalian cells (e.g., HEK293, CHO, CV-1, and Ltk<sup>-</sup> cells), insect cells, and the like.

The present invention also provides a diagnostic system, preferably in kit form, for assaying for the

presence of CRF-R protein, CRF-R polypeptide fragments or analogs, or CRF peptide in a fluid or tissue sample. A suitable diagnostic system includes, in an amount sufficient for at least one assay, a CRF-R protein (or  
5 polypeptide fragment thereof) and/or a subject antibody as a separately packaged immunochemical reagent. Instructions for use of the packaged reagent are also typically included.

10 "Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures,  
15 temperature, buffer conditions and the like.

In one embodiment, a diagnostic system for assaying for the presence or quantity of CRF-R (or more likely a fragment of CRF-R) in a vascular fluid sample,  
20 such as blood, plasma, or serum, or in a tissue sample, comprises a package containing at least one CRF-R protein or polypeptide fragment thereof of this invention. In addition, a diagnostic system containing at least one CRF-R (or polypeptide fragment thereof) can be used to detect  
25 the level of CRF peptide present in a vascular fluid sample or to detect the presence of an intracellular G-protein.

In another embodiment, a diagnostic system of  
30 the present invention for assaying for the presence or

amount of CRF-R or fragment or analog thereof in a sample includes an anti-CRF-R antibody composition of this invention.

5           In yet another embodiment, a diagnostic system of the present invention for assaying for the presence or amount of CRF-R or a CRF-R polypeptide in a sample contains at least one CRF-R (or polypeptide fragment thereof) and an anti-CRF-R antibody composition of this  
10   invention.

          In preferred embodiments, a diagnostic system of the present invention further includes a label or indicating means capable of signaling the formation of a  
15   complex containing a nucleic acid probe, protein, polypeptide, or antibody molecule of the present invention.

          Also contemplated are immunohistochemistry  
20   diagnostic systems for carrying out post-mortem diagnosis of mammalian tissue samples for the presence of CRF-R, which employ the anti-CRF-R antibodies described herein. For details on such diagnostic systems see, for example, Potter et al., PNAS, 89:4192-4296 (1992), incorporated  
25   herein by reference.

          In yet another embodiment of the present invention, a hybridization histochemistry diagnostic system is provided. This diagnostic system is useful for  
30   assaying for the presence or amount of nucleic acid

encoding CRF-R (e.g., cDNA or mRNA) in a sample (e.g., vascular fluid or cellular tissue). This diagnostic system employs at least one CRF-R encoding nucleic acid probe of this invention.

5

The word "complex" as used herein refers to the product of a specific binding reaction such as an antibody:antigen, receptor:ligand, protein:protein, or nucleic-acid-probe:nucleic-acid-target reaction.

10 Exemplary complexes are immunoreaction products and CRF:CRF-R complexes.

As used herein, the terms "label" and "indicating means" in their various grammatical forms  
15 refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal. Any label or indicating means can be linked to or incorporated in a nucleic acid probe, an expressed protein, polypeptide fragment, or antibody  
20 molecule that is part of an antibody or monoclonal antibody composition of the present invention, or used separately. These atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry.

25

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturation to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent  
30 labeling agents are fluorochromes such as fluorescein

isocyanate (FIC), fluorescein isothiocyanate (FITC),  
5-dimethylamine-1-naphthalenesulfonyl chloride (DANSC),  
tetramethylrhodamine isothiocyanate (TRITC), lissamine,  
rhodamine 8200 sulphonyl chloride (RB-200-SC), and the  
5 like. A description of immunofluorescence analytic  
techniques is found in DeLuca, "Immunofluorescence  
Analysis", in Antibody As a Tool, Marchalonis et al.,  
eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which  
is incorporated herein by reference.

10

In preferred embodiments, the indicating group  
is an enzyme, such as horseradish peroxidase (HRP),  
glucose oxidase, and the like. In such cases where the  
principal indicating group is an enzyme, additional  
15 reagents are required for the production of a visible  
signal. Such additional reagents for HRP include hydrogen  
peroxide and an oxidation dye precursor such as  
diaminobenzidine. An additional reagent useful with  
glucose oxidase is 2,2'-azino-di-(3-ethyl-benzthiazoline-  
20 G-sulfonic acid) (ABTS).

In another embodiment, radioactive elements are  
employed as labeling agents. An exemplary radiolabeling  
agent is a radioactive element that produces gamma ray  
25 emissions. Elements which emit gamma rays, such as  $^{124}\text{I}$ ,  
 $^{125}\text{I}$ ,  $^{126}\text{I}$ ,  $^{131}\text{I}$  and  $^{51}\text{Cr}$ , represent one class of radioactive  
element indicating groups. Particularly preferred is  $^{125}\text{I}$ .  
Another group of useful labeling means are those elements  
such as  $^{11}\text{C}$ ,  $^{18}\text{F}$ ,  $^{15}\text{O}$  and  $^{13}\text{N}$  which emit positrons. The  
30 positrons so emitted produce gamma rays upon encounters

with electrons present in the animal's body. Also useful is a beta emitter, such as  $^{32}\text{P}$ ,  $^{111}\text{indium}$  or  $^3\text{H}$ .

The linking of a label to a substrate, i.e.,  
5 labeling of nucleic acid probes, antibodies, polypeptides,  
and proteins, is well known in the art. For instance,  
antibody molecules can be labeled by metabolic  
incorporation of radiolabeled amino acids provided in the  
culture medium. See, for example, Galfre et al., Meth.  
10 Enzymol., 73:3-46 (1981). Conventional means of protein  
conjugation or coupling by activated functional groups are  
particularly applicable. See, for example, Aurameas et  
al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978),  
Rodwell et al., Biotech., 3:889-894 (1984), and U.S.  
15 Patent No. 4,493,795.

The diagnostic systems can also include,  
preferably as a separate package, a specific binding  
agent. A "specific binding agent" is a molecular entity  
20 capable of selectively binding a reagent species of the  
present invention or a complex containing such a species,  
but is not itself a polypeptide or antibody molecule  
composition of the present invention. Exemplary specific  
binding agents are second antibody molecules (e.g.,  
25 anti-Ig antibodies), complement proteins or fragments  
thereof, S. aureus protein A, and the like. Preferably  
the specific binding agent binds the reagent species when  
that species is present as part of a complex.

In preferred embodiments, the specific binding agent is labeled. However, when the diagnostic system includes a specific binding agent that is not labeled, the agent is typically used as an amplifying means or reagent.

5 In these embodiments, the labeled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a reagent species-containing complex.

10 The diagnostic kits can be used in an "ELISA" format to detect the quantity of CRF, CRF-R, or CRF:CRF-R complex in a vascular fluid sample such as blood, serum, or plasma or in a mammalian tissue sample. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an  
15 antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by  
20 D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patent Nos. 3,654,090, No. 3,850,752; and No. 4,016,043, which are all incorporated herein by reference.

25 Thus, in preferred embodiments, CRF-R protein, a CRF-R polypeptide fragment thereof, a polyclonal anti-CRF-R antibody, or a monoclonal anti-CRF-R antibody is affixed to a solid matrix to form a solid support that comprises a package in the subject diagnostic systems. A  
30 reagent is typically affixed to a solid matrix by



adsorption from aqueous medium, although other modes of affixation applicable to proteins and polypeptides well known to those skilled in the art can be used.

5            Useful solid matrices are also well known in the art. Such materials are water insoluble and include cross-linked dextran (available from Pharmacia Fine Chemicals; Piscataway, NJ); agarose; beads of polystyrene about 1 micron to about 5 millimeters in diameter  
10 (available from Abbott Laboratories; North Chicago, IL); polyvinyl chloride; polystyrene; cross-linked polyacrylamide; nitrocellulose- or nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from  
15 polystyrene or polyvinylchloride.

          The reagent species, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid  
20 dispersion or as a substantially dry power, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microtiter plate and one or more buffers  
25 can also be included as separately packaged elements in this diagnostic assay system.

          The packaging materials contemplated herein in relation to diagnostic systems are those customarily  
30 utilized in diagnostic systems. The term "package" refers

to a solid matrix or material such as glass, plastic (e.g., polyethylene, polypropylene and polycarbonate), paper, foil, and the like, capable of holding within fixed limits a diagnostic reagent such as a protein, polypeptide  
5 fragment, antibody or monoclonal antibody of the present invention. Thus, for example, a package can be a bottle, vial, plastic or plastic-foil laminated envelope container, or the like, used to contain a diagnostic reagent. Alternatively, the container used can be a  
10 microtiter plate well to which microgram quantities of a diagnostic reagent have been operatively affixed, i.e., linked so as to be capable of being immunologically bound by an antibody or polypeptide to be detected.

15 In normal individuals, the levels of CRF can vary from about 1 to 28 picograms per milliliter of vascular fluid. However, during the last trimester of pregnancy, it has been found that there is a tendency for CRF levels to prematurely increase. It is believed that  
20 this increase is associated with pregnancy-induced hypertension. Monitoring the change in the level of CRF could facilitate the prediction of the possibility of premature labor, which can be avoided by appropriate treatment.

25

Thus, by monitoring the level of CRF, an abnormal increase indicative of a potential pathological problem in pregnancy can be detected at an early stage. Because normal hypertension is now believed to be either  
30 caused (or accompanied by) a higher CRF/"CRF-binding

protein" ratio than normal, monitoring the level of CRF facilitates the prediction of particular patients who are predisposed to such diseases, and permits therapeutic intervention--as for example by administering dosages of  
5 CRF-R protein or polypeptide fragments thereof. By the administration of CRF-R or fragments thereof to treat such pregnancy related disorders, CRF levels can be returned to normal, thus facilitating the normal growth of the fetus.

10           The present invention contemplates various immunoassay methods for determining the amount of CRF-R in a biological fluid or tissue sample using a CRF-R, a polypeptide fragment thereof (including immunologic fragments, i.e., fragments capable of generating an immune  
15 response, as more fully described in Harlowe and Lane, Antibodies: A Laboratory Manual, p. 76 (Cold Spring Harbor Laboratory, 1988)), an anti-CRF-R polyclonal or monoclonal antibody of this invention as an immunochemical reagent to form an immunoreaction product whose amount relates,  
20 either directly or indirectly, to the amount of CRF-R in the sample. Also contemplated are immunoassay methods for determining the amount of CRF peptide in a biological fluid sample using a CRF-R or a polypeptide fragment thereof as a reagent to form a product whose amount  
25 relates, either directly or indirectly, to the amount of CRF in the sample.

          Various well-known heterogenous and homogenous protocols, either competitive or noncompetitive, solution-  
30 phase or solid-phase, can be employed in performing assay

methods of the present invention. Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures in which an immunochemical reagent of this invention can be used to  
5 form an immunoreaction product whose amount relates to the amount of CRF-R or CRF present in a body sample.

In one embodiment, the detection of CRF-R protein or polypeptide fragments in a body sample is  
10 utilized as a means to monitor the fate of therapeutically administered CRF-R or polypeptide fragments according to the therapeutic methods disclosed herein.

Also contemplated are immunological assays  
15 capable of detecting the formation of immunoreaction product formation without the use of a label. Such methods employ a "detection means", which means are themselves well-known in clinical diagnostic chemistry. Exemplary detection means include biosensing methods based  
20 on detecting changes in the reflectivity of a surface, changes in the absorption of an evanescent wave by optical fibers, changes in the propagation of surface acoustical waves, and the like.

25 The present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically compatible carrier together with a CRF-R protein, CRF-R polypeptide fragment,  
30 or anti-CRF-R antibody, as described herein, dissolved or

dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

5

As used herein, the terms "pharmaceutically acceptable", "physiologically compatible" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset, and the like.

15

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well known in the art. Typically such compositions are prepared as injectables either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspension, in liquid prior to use can also be prepared. The preparation can also be emulsified.

20

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, as well as combinations of any two or more thereof. In addition, if desired, the composition can contain minor amounts of

25

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auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like, which enhance the effectiveness of the active ingredient.

5           The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are  
10   formed with inorganic acids such as, for example, hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic  
15   acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid, and the like.

          Salts formed with the free carboxyl groups can  
20   also be derived from inorganic bases such as, for example, sodium hydroxide, ammonium hydroxide, potassium hydroxide, and the like; and organic bases such as mono-, di-, and tri-alkyl and -aryl amines (e.g., triethylamine, diisopropyl amine, methyl amine, dimethyl amine, and the  
25   like) and optionally substituted ethanolamines (e.g., ethanolamine, diethanolamine, and the like).

          Physiologically tolerable carriers are well known in the art. Exemplary liquid carriers are sterile  
30   aqueous solutions that contain no materials in addition to

the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary additional liquid phases include glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

As previously indicated, administration of the CRF-Rs or polypeptide fragments thereof is effective to reduce vascular fluid CRF levels or high ACTH levels in mammals caused by excessive CRF, which is referred to herein as "CRF-induced ACTH release." In this manner, the CRF-Rs are useful in treating high cortisol (i.e., glucocorticoids) levels which are associated with hypercortisolemia, Cushing's Disease, alcoholism, anorexia nervosa and similar diseases. Likewise, these CRF-Rs are considered to have utility in combatting pituitary tumors that produce CRF--particularly in maintaining stability in a patient until such a tumor can be surgically removed.

In accordance with the present invention, CRF-RB<sub>1</sub> has surprisingly been found to be abundantly expressed in the heart. In the isolated perfused heart, the

addition of CRF into the left atrium induces a prolonged dilatory effect on coronary arteries, transiently produces a positive inotropic effect, and stimulates the secretion of atrial natriuretic peptide (Saitoh et al., Gen. Pharmac. (England) 21:337-342 (1990); and Grunt et al., Horm. Metab. Res. (Germany) 24:56-59 (1992)). The surprising finding of CRF-RB<sub>1</sub> expression in the blood vessels of the heart raises the possibility that CRF (or other natural or pharmacologic ligands for this receptor) might regulate cardiac perfusion. Furthermore, it is expected that other vascular beds, such as the superior mesenteric, known to be dilated by CRF and related ligands, will also be found to be regulated by CRF and receptors therefor.

15

Accordingly, since it is known that CRF has a number of biological effects in the heart, it is contemplated that the CRF-R proteins, fragments thereof, or agonists/antagonists thereof (e.g., anti-CRF-R antibodies), can be effectively used to selectively modulate the action of CRF on the heart, particularly in methods to increase the level of CRF that can act on the heart blood vessels to maintain patency.

25

In accordance with the present invention, the CRF-RB gene has also been found to be expressed in the gastrointestinal tract, as shown, for example, by the presence of CRF-RB<sub>1</sub> mRNA in the submucosal and deeper regions of the duodenum. Accordingly, the CRF-RB<sub>1</sub> receptor may mediate some of the direct stimulatory effects of CRF

30



on the GI tract that have been described. For example, CRF acts on the gut in vitro to depolarize myenteric neurons in the small intestine (Hanani and Wood, Eur. J. Pharmacol. (Netherlands) 211:23-27 (1992)). In vivo studies with intravenously administered CRF and CRF antagonists are consistent with a direct effect of CRF to control gastric emptying and intestinal motility (Williams et al., Am. J. Physiol. 253:G582-G586 (1987); Lenz, H.J., Horm. Metab. Res. Suppl. 16:17-23 (1987); and Sheldon et al., Regul. Pept. 28:137-151 (1990)). In addition, CRF immunostaining is present at many levels of the GI tract (Nieuwenhuyzen-Kruseman et al., Lancet 2:1245-1246 (1982); Petrusz et al., Federation Proc. 44:229-235 (1985); and Kawahito et al., Gastroenterology 106:859-865 (1994)).

15

Thus, CRF-Rs (e.g., CRF-RB), fragments thereof, or agonists/antagonists thereof (e.g., anti-CRF-R antibodies), are contemplated for use in treating gastrointestinal disorders, such as irritable bowel syndrome. In addition, CRF antagonists that are selective for CRF-RB are also contemplated for use in therapeutic methods to treat irritable bowel syndrome.

In addition, the presence of CRF-RB in the epididymis may enable local communication with spermatozoa, which are reported to possess immunoreactive CRF (Yoon et al., Endocrinology 122:759-761 (1988)). Thus, CRF-Rs are also contemplated for use in treating fertility disorders.

30

The CRF-R proteins and fragments thereof are also useful to treat abnormalities, such as, for example, preeclampsia (toxemia of pregnancy), which occur during pregnancy; for example, they can be used to reduce pregnancy-induced complications and increased CRF levels which can otherwise result in excessive release of ACTH. In addition, CRF-R proteins or fragments thereof can be administered to sequester CRF from vascular fluid, thereby reducing the ratio of CRF/"CRF-binding protein" present in a patient, wherein it is beneficial to reduce the levels of free CRF (i.e., CRF not bound to CRF-BP) in the vascular fluid sample. CRF-binding protein (CRF-BP) is an extracellular serum protein described in Potter et al., supra. The IV administration of CRF-Rs may also be employed in certain instances to modulate blood pressure and thereby combat hypotension.

Since CRF is a known modulator of the immune system, it is contemplated that the administration of CRF-R protein or fragments thereof may be useful to locally treat, i.e., by direct injection into the affected joint, arthritis and other similar ailments. CRF is known to have a number of biological effects on the pituitary, and accordingly, the CRF-R proteins can be used to modulate the action of CRF on the pituitary. Furthermore, it is well known that CRF has a number of biological effects in the brain; therefore, it is contemplated that the CRF-R proteins can be effectively used to modulate the action of CRF on the brain, particularly with respect to control of appetite, reproduction, growth, anxiety, depression, fever

and metabolism, as well as the regulation of blood pressure, heart rate, blood flow, and the like.

Thus, the present invention provides for a  
5 method for modulating the action of CRF in mammals comprising administering a therapeutically effective amount of a physiologically acceptable composition containing CRF-R protein or polypeptide fragment of the present invention. In addition, the stimulation of ACTH  
10 release by CRF can be enhanced by transfecting the subject with a tissue specific CRF-encoding construct.

In another embodiment, the present invention provides a method for treating a pregnancy-related  
15 pathological disorder in mammals comprising administering a therapeutically effective amount of a physiologically acceptable composition containing a CRF-R protein or polypeptide fragment of the present invention, said amount being effective to sequester CRF, thereby producing a  
20 CRF/"CRF-binding protein" ratio within the normal range for a pregnant female.

Also, as earlier indicated, the administration of anti-CRF-R antibodies described herein is effective to  
25 modulate the biological effect of CRF-Rs when administered in vivo. For example, an anti-CRF-R antibody of this invention can be used in the above-described mammalian therapeutic methods to: neutralize or counteract the effect of CRF-R, increase the level of free CRF (e.g., CRF  
30 not bound by CRF-R), decrease CRF-induced ACTH release, or

decrease the level of ACTH-induced glucocorticoids in a subject. Because increasing the level of free CRF increases the level of CRF-induced ACTH release, which increases glucocorticoid production, these therapeutic  
5 methods are useful for treating certain physiological conditions where increasing the level of glucocorticoids in a patient's vascular fluid is therapeutically effective, such as conditions of inflammation or Addison's Disease, and the like.

10

Administration of antibodies for this purpose would be carried out along the lines and in amounts generally known in this art, and more particularly along the lines indicated herein with respect to administration  
15 of the protein itself.

As described herein, a therapeutically effective amount is a predetermined amount calculated to achieve the desired effect, e.g., to decrease the amount of CRF, ACTH,  
20 or decrease the CRF/"CRF-binding protein" ratio in a patient. The required dosage will vary with the particular treatment and with the duration of desired treatment; however, it is anticipated that dosages between about 10 micrograms and about 1 milligram per kilogram of  
25 body weight per day will be used for therapeutic treatment. It may be particularly advantageous to administer such compounds in depot or long-lasting form as discussed hereinafter. A therapeutically effective amount is typically an amount of a CRF-R protein or polypeptide  
30 fragment thereof that, when administered in a

physiologically acceptable composition, is sufficient to achieve a plasma concentration of from about 0.1 mg/ml to about 100 mg/ml, preferably from about 1.0 mg/ml to about 50 mg/ml, more preferably at least about 2 mg/ml and  
5 usually 5 to 10 mg/ml. Antibodies are administered in proportionately appropriate amounts in accordance with known practices in this art.

The level of ACTH present in a patient,  
10 particularly in the plasma, can be readily determined by routine clinical analysis. In addition, changes in ACTH levels can be monitored during a treatment regimen to determine the effectiveness of the administered CRF-R protein or polypeptide fragment over time.

15

Thus, the present therapeutic method provides an in vivo means for decreasing ACTH levels in a subject displaying symptoms of elevated serum ACTH, or is otherwise at medical risk by the presence of serum ACTH,  
20 wherein it is beneficial to reduce the levels of ACTH. In addition, the present therapeutic method provides an in vivo means for decreasing ACTH-induced cortisol levels (e.g., glucocorticoids) in a human patient displaying symptoms of elevated serum cortisol.

25

Likewise, the level of CRF present in a patient, particularly in the plasma, can be readily determined by the diagnostic methods and kits provided herein and readily manipulated by administering CRF-R, analogs  
30 thereof, or anti-CRF-R antibodies.

Thus, the present therapeutic method provides an in vivo means for decreasing the CRF/CRF-BP ratio in a subject displaying symptoms of elevated serum CRF/CRF-BP levels, or is otherwise at medical risk by the presence of an elevated serum CRF/CRF-BP ratio, wherein it is beneficial to reduce the levels of free CRF (i.e., CRF not bound to CRF-BP) in the vascular fluid sample.

CRF-R protein(s) (or functional fragments thereof) should be administered under the guidance of a physician. Pharmaceutical compositions will usually contain the protein in conjunction with a conventional, pharmaceutically-acceptable carrier. For treatment, substantially pure synthetic CRF-R or a nontoxic salt thereof, combined with a pharmaceutically acceptable carrier to form a pharmaceutical composition, is preferably administered parenterally to mammals, including humans, either intravenously, subcutaneously, intramuscularly, percutaneously, e.g. intranasally, or intracerebroventricularly; oral administration is possible with an appropriate carrier.

Therapeutic compositions containing CRF-R polypeptide(s) of this invention are preferably administered intravenously, as by injection of a unit dose, for example. The term "unit dose," when used in reference to a therapeutic composition of the present invention, refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a

predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, i.e., carrier, or vehicle.

5               Compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the  
10 active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed  
15 herein and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more intervals by a subsequent injection or other  
20 administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

25               As an aid to the administration of effective amounts of a CRF-R polypeptide, a diagnostic method of this invention for detecting a CRF-R polypeptide in the subject's blood is useful to characterize the fate of the administered therapeutic composition.

It may also be desirable to deliver CRF-R over prolonged periods of time, for example, for periods of one week to one year from a single administration, and slow release, depot or implant dosage forms may be utilized.

- 5 For example, a dosage form may contain a pharmaceutically acceptable non-toxic salt of the compound which has a low degree of solubility in body fluids, for example, an acid addition salt with the polybasic acid; a salt with a polyvalent metal cation; or combination of the two salts.
- 10 A relatively insoluble salt may also be formulated in a gel, for example, an aluminum stearate gel. A suitable slow release depot formulation for injection may also contain CRF-R or a salt thereof dispersed or encapsulated in a slow degrading, non-toxic or non-antigenic polymer
- 15 such as a polylactic acid/polyglycolic acid polymer, for example, as described in U.S. Pat. No. 3,773,919. These compounds may also be formulated into silastic implants.

- As additional examples of the utility of
- 20 invention compositions, nucleic acids, receptors and/or antibodies of the invention can be used in such areas as the diagnosis and/or treatment of CRF-dependent tumors, enhancing the survival of brain neurons, inducing abortion in livestock and other domesticated animals, inducing
- 25 twinning in livestock and other domesticated animals, and so on.

- In addition, invention cDNAs described herein encoding CRF-Rs (e.g., CRF-RA and CRF-RB) can be used to
- 30 isolate genomic clones encoding the respective CRF-R gene.



The 5' regulatory region of the isolated CRF-R gene can be sequenced to identify tissue-specific transcription elements (i.e., promoter). The tissue-specific CRF-R promoters obtained are useful to target various genes to cells that normally express CRF-Rs. For example, an adenovirus vector, having DNA encoding a cytotoxic protein and a tissue-specific CRF-R promoter for pituitary corticotropic cells, can be used as means for killing pituitary corticotropic tumor cells.

10

The invention will now be described in greater detail by reference to the following non-limiting examples.

#### EXAMPLES

15

Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1982); Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1989); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987); or Harlowe and Lane, Antibodies: A Laboratory Manual, p. 76 (Cold Spring Harbor Laboratory, 1988).

30

Double-stranded DNA was sequenced by the dideoxy chain termination method using the Sequenase reagents from US Biochemicals. Comparison of DNA sequences to databases was performed using the FASTA program [Pearson and Lipman, 5 Proc. Natl. Acad. Sci. USA 85: 2444-2448 (1988)]. Tyr-ovine CRF used for iodination was purchased from Peninsula.

#### Example 1

##### 10 Isolation of cDNA encoding a human CRF-R

A cDNA library of approximately  $1.5 \times 10^6$  independent clones from human pituitary corticotrope adenoma (Cushing's Tumor) cells was constructed in the 15 mammalian expression vector, pcDNA1, and screened using an expression cloning approach [Gearing et al., EMBO J. 8, 3667-3676 (1989)] based on the ability of single transfected cells to detectably bind labeled  $^{125}\text{I}$ -Tyr-ovine CRF. Binding was assessed by performing the transfections 20 and binding reactions directly on chambered microscope slides, then dipping the slides in photographic emulsion, developing the slides after 3-4 days exposure, and analyzing them under a microscope. The possibility of detecting expressed CRF Binding Protein, CRF-BP, rather 25 than authentic CRF-R, was minimized by the selection of an ovine CRF related tracer known to have high affinity for the receptor but low affinity for CRF-BP. Cells which had been transfected with CRF receptor cDNA, and consequently bound radioactive CRF, were covered with silver grains.

Polyadenylated RNA was prepared from human pituitary corticotrope adenoma cells. Corresponding cDNA was synthesized and ligated into the plasmid vector pcDNA1 using non-palindromic BstXI linkers and used to transform 5 MC1061/P3 cells, yielding a library of approximately  $1.5 \times 10^6$  primary recombinants. The unamplified cDNA library was plated at approximately 5000 clones per 100 mm plate. The cells were then scraped off the plates, frozen in glycerol, and stored at  $-70^\circ\text{C}$ .

10

Mini-prep DNA was prepared from each pool of 5,000 clones using the alkaline lysis method [Maniatis et al. Molecular Cloning (Cold Spring Harbor Laboratory (1982))]. Approximately 1/10 of the DNA from a mini-prep 15 (10 ml of 100 ml) was transfected into COSM6 cells, and the cells screened for the capacity to bind iodinated Tyr ovine CRF.

More specifically,  $2 \times 10^5$  COS cells were plated 20 on chambered microscope slides (1 chamber - Nunc) that had been coated with 20 mg/ml poly-D-lysine and allowed to attach for at least 3 hours in DMEM and 10% Fetal Calf Serum (complete medium). Cells were subjected to DEAE-Dextran mediated transfection as follows. 1.5 ml of 25 serum-free Dulbecco's Modified Eagle's medium (DMEM) containing 100 mM chloroquine was added to the cells. DNA was precipitated in 200 ml DMEM/chloroquine containing 500 mg/ml DEAE-Dextran, then added to the cells. The cells were incubated at  $37^\circ\text{C}$  for 4 hours, then the media was 30 removed and the cells were treated with 10% DMSO in HEPES

buffered saline for 2 minutes. The 10% DMSO was removed, and fresh complete media was added and the cells assayed for binding 2 days later.

5 Transfected cells prepared as described above were washed twice with HEPES buffered saline (HDB) containing 0.1% ovalbumin, then incubated for 90 minutes at 22°C in 0.7 ml HDB, 0.1% ovalbumin containing  $10^6$  cpm  $^{125}$ I-Tyr-ovine CRF (approximately 1 ng, 300 pM). The cells  
10 were then washed 3X with cold HDB, 0.1% ovalbumin, and 2X with cold HDB, then fixed for 15 minutes at 22°C in 2.5% glutaraldehyde/HDB and washed 2X with HDB. The chambers were then peeled off the slides, and the slides dehydrated in 95% ethanol, dried under vacuum, dipped in NTB2  
15 photographic emulsion (Kodak) and exposed in the dark at 4°C for 3-4 days. Following development of the emulsion, the slides were dehydrated in 95% ethanol, stained with eosin and coverslipped with DPX mountant (Electron Microscopy Sciences). The slides were analyzed under  
20 darkfield illumination using a Leitz microscope.

Successive subdivision of a positive pool generated a single clone that demonstrated high affinity CRF binding ( $K_d = 3.3 \pm 0.45$  nM) when present in COSM6 cell  
25 membranes. The clone containing sequence encoding CRF-receptor is referred to herein as "hctCRFR" and has been deposited with ATCC under accession number 75474, and the receptor encoded thereby is referred to herein as hCRF-RA<sub>1</sub>.

A phage lZapII library was also synthesized from the same human Cushing's tumor cDNA described above using NotI/EcoRI adapters. A 1.2 kb PstI fragment in the CRF-R coding region of clone "hctCRFR" was used to screen the lZapII library at high stringency using standard methods. Of three positive clones identified, two were sequenced and found to contain full length CRF-R cDNA without introns. The clones are labeled "CRF-R1" (also referred to herein as hCRF-RA<sub>1</sub>) and "CRF-R2" (also referred to herein as hCRF-RA<sub>2</sub>), portions of which are set forth in SEQ ID NO:1 and SEQ ID NO:3, respectively. Clone CRF-R1 (i.e., hCRF-RA<sub>1</sub>) contains a 2584 bp insert with a 1245 bp open reading frame encoding a 415 amino acid CRF-R protein. Clone CRF-R2 (also referred to herein as hCRF-RA<sub>2</sub>), is an alternatively spliced variant sequence of CRF-R1 (i.e., hCRF-RA<sub>1</sub>) that has the 29 amino acids set forth in SEQ ID NO:4 inserted between amino acids 145-146 of SEQ ID NO:2.

## Example 2

### Expression of CRF Receptor mRNA

Using well-known autoradiographic methods for binding labelled CRF to various frozen tissue sections, the native CRF receptor has been detected and shown to vary dynamically in the pituitary and various brain regions in experimental animals and in human beings where it is altered in pathologic conditions including Alzheimer's Disease and severe melancholic depression. Furthermore, receptors have been detected in the periphery

in organs such as the adrenal, ovary, placenta, gastrointestinal tract and the red pulp, macrophage rich area of the spleen and in sites of inflammation presumably corresponding to the actions of CRF within those tissues.

5

A Northern-blot assay was conducted by size-fractionating poly(A)<sup>+</sup>-RNA (derived from rat brain, rat pituitary, rat heart, and mouse AtT20 corticotropic cells) on a denaturing formaldehyde agarose gel and transferring  
10 the RNA to nitrocellulose paper using standard methods. The nitrocellulose paper blot was prehybridized for 15 minutes at 68°C in QuikHyb<sup>®</sup> hybridization solution (Stratagene, La Jolla, CA) and 100 mg/ml salmon sperm DNA. Next, the blot was hybridized in the same solution at 68°C  
15 for 30 minutes to a "hctCRFR"-derived randomly primed (Amersham, Arlington Heights, IL) 1.3 Kb PstI cDNA fragment that contained the majority of the cDNA region of CRF-R1 (i.e., hCRF-RA<sub>1</sub>). The blot was washed twice at 21°C in 2X SSPE and 0.15% Sodium Dodecyl Sulfate (SDS) for 15  
20 minutes. Next, the blot was washed twice at 60°C in 0.2 X SSPE and 0.1% SDS for 30 minutes. An autoradiogram of the nitrocellulose paper blot was developed using standard methods.

25 The results of the Northern-blot assay revealed the presence of a 2.7 Kb CRF-R mRNA transcript in rat brain, rat pituitary, and in mouse AtT20 corticotropic cells. CRF-R mRNA was not detected in the heart tissue sample.

30

Example 3Pharmacologic characteristics of hctCRFR  
transiently expressed in COSM6 cells

5            Approximately  $10^6$  COSM6 cells were transfected  
with either hctCRFR or rGnRHR (rat gonadotropin releasing  
hormone receptor) by the DEAE-dextran method and grown in  
150 mm tissue culture dishes. Two days after  
transfection, the cells were washed twice with 1 ml HDB  
10 and were detached by incubation for 15 min at room  
temperature in 0.5 mM EDTA in HDB. After pelleting, the  
cells were washed twice with HDB, and then homogenized in  
5% sucrose (16ml/150mm dish). The homogenate was  
centrifuged at 600 X g for 5 minutes, and the resulting  
15 supernatant was centrifuged at 40,000 X g for 20 minutes.  
The resulting pellet (containing crude membranes) was  
resuspended at 1-4mg/ml in 10% sucrose, and used in a  
competitive radioreceptor assay to measure binding to the  
CRF-R as described in Perrin et al., Endoc., 118:1171  
20 (1986).

Membrane homogenates (10-24 mg) were incubated  
at room temperature for 90 minutes with 100,000 cpm  $^{125}\text{I}$ -  
(Nle<sup>21</sup>, Tyr<sup>32</sup>)-ovine CRF (1 mg CRF was iodinated by  
25 chloramine T oxidation to a specific activity of 2,000  
Ci/mmol; iodinated CRF was purified by HPLC) and  
increasing concentrations of unlabeled rat/human (r/h)  
CRF. The iodinated CRF and unlabeled r/h CRF were both  
diluted in 20 mM HEPES, 0.1% BSA, 10% sucrose, 2 mM EGTA  
30 to a final, pH 7.5 in a final volume of 200 ml and

containing  $\text{MgSO}_4$  to a final concentration of 10mM. The reaction was terminated by filtration through GF/C (Whatman) filters, prewetted with 1% BSA, 10mM HEPES, pH 7.5. The filters were washed 4 times with 1 ml 0.1% BSA, 50 mM Tris, pH 7.5. Filter-bound radioactivity, indicating the presence of CRF-R:  $^{125}\text{I}$ -(Nle<sup>21</sup>, Tyr<sup>32</sup>)-ovine CRF complex, was determined by g-scintillation counting.

The results from an assay for the displacement of  $^{125}\text{I}$ -(Nle<sup>21</sup>, Tyr<sup>32</sup>)-ovine CRF by unlabeled human/rat CRF (r/h CRF) are shown in Figure 1. The results show that native r/h CRF is able to displace labeled ovine CRF in a dose-dependent manner from cells transfected with hctCRFR, but not from cells transfected with rGnRHR. This indicates that the hctCRFR clone encodes a receptor that displays pharmacologic specificity characteristic of a physiologically relevant CRF-receptor (i.e., CRF-RA<sub>1</sub>).

#### Example 4

##### Assay of CRF-R mediated stimulation of intracellular cAMP levels

To determine the possible linkage of CRF-R to multiple signaling pathways, the ability of CRF-R to stimulate cAMP formation in CRF-R-expressing COSM6 cells was investigated. To ensure that changes in cAMP levels were not influenced by alterations in cAMP phosphodiesterase, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to the medium. COSM6 cells were trypsinized 24 hrs following transfection



with either ctCRFR or rGnRHR in 150 mm dishes and were replated in 24-well plates (Costar) and allowed to express the receptors for another 24 hrs in 10% FCS, DMEM.

5           On the day of the stimulation, the medium was changed to 0.1% FCS, DMEM at least 2 hrs before a 30 minute preincubation with 0.1 mM IBMX or medium. Test ligands (i.e., r/h CRF, sauvagine, salmon calcitonin, Vasoactive intestinal peptide (VIP), growth hormone releasing factor (GRF) were added in 0.1% BSA, 0.1% FCS, 10       DMEM, and stimulation was carried out for 30 minutes at 37 °C, 7.5% CO<sub>2</sub>. The medium was removed and the cells were extracted overnight with 1 ml ice-cold 95% EtOH-0.1M HCl at -20 °C. Cyclic AMP (cAMP) levels were determined in 15       duplicate from triplicate wells by RIA kit (Biomedical Technologies, Stoughton, MA) following the manufacturer's protocol.

          The results are shown in Figures 2A, 2B and 2C. 20       Figures 2A and 2B show that COSM6 cells transfected with the cloned hctCRFR respond to CRF with an approximately 10-20 fold increase in intracellular cAMP over basal cAMP levels. Several unrelated peptides have no effect on cyclic AMP levels in the receptor transfected cells. 25       Figure 2C shows that the CRF antagonist, a helical (9-41) CRF, blocks the induction of cyclic AMP by r/h CRF.

#### Example 5

#### Isolation of cDNA encoding a rat CRF-R

Adult Sprague-Dawley rat whole brain poly (A)+ RNA was used for the synthesis of a cDNA library. Double-stranded cDNA was ligated to EcoRI-NotI adaptors (Pharmacia/LKB) and cDNAs greater than 2 kilobase pair (kb) were ligated into the lZAPII vector (Stratagene, La Jolla, CA). The library was amplified once and approximately  $7 \times 10^5$  clones were screened by hybridization with the 1.2kb PstI fragment of CRF-R1 (e.g., CRF-RA<sub>1</sub>) using standard methods. One of the positive clones identified was sequenced and found to contain full length CRF-R cDNA. The positive clone was labeled rat brain CRF-R (rbCRF-RA) and contains an approximate 2500 base pair (bp) insert with a 1245 bp open reading frame encoding a 415 amino acid CRF-R protein. The cDNA and amino acid sequences corresponding to "rbCRF-RA" are set forth in SEQ ID NOs: 5 and 6, respectively.

#### Example 6

##### Isolation of genomic DNA encoding a mouse CRF-RB<sub>1</sub>

20

Approximately  $7 \times 10^6$  clones of a mouse phage genomic library (obtained from Stratagene, La Jolla, CA) were screened by hybridization with a probe comprising nucleotides 204-1402 of rat CRF-RA (see SEQ ID NO:5) using standard methods. Thus, hybridization was carried out in 5X SSPE, 5X Denhardt's solution, and 0.5% SDS for 16 hours at 60°C. The filters were washed twice at room temperature with 2X SSC, 0.1% SDS, then washed twice at 60°C with 2X SSC, 0.1% SDS.

30

One of the positive clones identified was sequenced and found to contain an open reading frame encoding a partial CRF-RB<sub>1</sub> sequence derived from transmembrane domains 3 through 4 of CRF-RB<sub>1</sub>. The positive  
5 clone was labeled mouse CRF-RB<sub>1</sub> (mCRF-RB<sub>1</sub>) and contains two exons, interrupted by an intron of about 450 nucleotides. The two exons combine to produce a 210 base pair (bp) open reading frame encoding a 70 amino acid portion of a novel CRF-RB<sub>1</sub> protein. The cDNA and amino acid sequences  
10 corresponding to "mCRF-RB<sub>1</sub>" are set forth in SEQ ID NOs: 7 and 8, respectively.

Upon further sequencing of clone mCRF-RB<sub>1</sub>, a third exon was revealed containing an additional 78 base  
15 pairs (corresponding to nucleotides 895-972 of SEQ ID NO:9) of an open reading frame encoding the CRF-RB<sub>1</sub> protein.

#### Example 7

##### 20 Isolation of cDNA encoding a mouse CRF-RB<sub>1</sub>

In order to obtain the cDNA corresponding to the new mouse CRF-RB<sub>1</sub> receptor, a mouse heart phage library was screened by hybridization. Approximately  $1.2 \times 10^6$  phage  
25 plaques of an amplified oligo-dt primed mouse heart cDNA library in the lambda zap II vector (Stratagene) were screened by hybridization. A probe for the CRF-RB<sub>1</sub> cDNA was prepared by PCR using [<sup>32</sup>P]-dCTP and the following primers:  
30 sense: 5' CTGCATCACCACCATCTTCAACT 3' (SEQ ID NO:11); and

antisense: 5' AGCCACTTGCGCAGGTGCTC 3' (SEQ ID NO:12).

The template used in generating the probe was plasmid DNA corresponding to one exon of CRF-RB<sub>1</sub> extending from amino acids 206 to 246 of SEQ ID NO:10. PCR amplification was carried out for 30 cycles (denatured at 94°C for 1 minute, annealed at 55°C for 2 minutes and extended at 72°C for 3 minutes) to yield a 123 bp product corresponding to nucleotides 693-815 of SEQ ID NO:9.

10

For hybridization screening, the plaques were lifted onto nylon membranes, then denatured, neutralized and rinsed. The membranes were prehybridized at 42°C in 0.6M NaCl, 60mM sodium citrate, 4X Denhardt's, 40mM sodium phosphate, pH 6.5, 170 mg/ml salmon sperm DNA, 0.1% SDS, 20% formamide. The membranes were then hybridized at 42°C in the same solution plus 50% dextran sulfate with approximately 10<sup>6</sup> cpm of labeled probe per ml of solution. After hybridization, the filters were washed with 0.3M NaCl, 30mM sodium citrate, 0.1% SDS once at room temperature, twice at 42°C, and twice at 50°C.

A positive plaque was isolated and purified in the next round of plaque hybridization. Helper phage R408 (Biorad) was used for in vivo excision of the lambda zap II clone. The cloned receptor was sequenced on both strands by the dideoxy chain-termination method using the Sequenase kit (United States Biochemical). The clone encoding mouse CRF-RB<sub>1</sub> contained a 2.2 kb insert that included a 1293 base pair (bp) open reading frame encoding

30

a protein of 431 amino acids. The full-length CRF-RB<sub>1</sub> receptor cDNA was subcloned into the expression vector pcDNA1 (Invitrogen) using the EcoRI restriction enzyme to produce the plasmid pCRF-RB<sub>1</sub>.

5

Alignments of the nucleotide and amino acid sequences were carried out using the Jotun-Hein weighted method and the PAM250 residue weight table, respectively. CRF-RB<sub>1</sub> has 70% homology at the nucleotide level and 68%  
10 homology at the amino acid level, to CRF-RA<sub>1</sub>. Figures 3A and 3B present the comparison between the amino acid sequences of CRF-RB<sub>1</sub> and CRF-RA<sub>1</sub>. The alignment was selected to maximize the regions of similarity. There is a putative signal peptide and five putative N-  
15 glycosylation sites in the N-terminal domain in CRF-RB<sub>1</sub>, as there are in CRF-RA<sub>1</sub>. When comparing CRF-RB<sub>1</sub> to CRF-RA<sub>1</sub>, there is 79% similarity in the seven transmembrane domains (TMD) and 84% similarity in the intracellular loops and the intracellular tail. Yet there is only 60% identity in  
20 the extracellular loops (ECL), and 40% identity in the N-terminal domain, which has an additional sixteen amino acids.

All but one of the putative phosphorylation  
25 sites in CRF-RA<sub>1</sub> are found in CRF-RB<sub>1</sub>, the missing one being in the C-terminus. CRF-RB<sub>1</sub> also has an extra cysteine in a region of the N-terminus which may be within a cleaved putative signal peptide, as well as an extra cysteine at the junction of extracellular loop-1 and

transmembrane domain-3, so that the residue may fall within this transmembrane domain.

#### Example 8

5                   Pharmacologic characteristics of CRF-RB<sub>1</sub>  
                    transiently expressed in COSM6 cells

Using the methods described in Example 3, a radioreceptor assay was conducted. Approximately 10 mg of  
10 pCRF-RB<sub>1</sub> plasmid DNA was transfected into COSM6 cells using the DEAE dextran method. Two days later, the cells were detached and crude membrane fractions were prepared and used to measure binding by competitive displacement of  
125I-(Nle21,Tyr32)-ovine CRF. In order to calculate a K<sub>d</sub>,  
15 the displacement data were analyzed using the Ligand program of Munson and Rodbard (1980), Anal. Biochem., 107:220-239.

The cloned CRF-RB<sub>1</sub> binds CRF with high affinity  
20 as determined by the competitive displacement of bound radioligand. From six experiments, the dissociation constant was determined to be approximately K<sub>d</sub> = 1.3±0.2 nM (see Figure 4). The binding is specific because the peptides GRF and VIP do not displace the bound  
25 radioligand. In addition, the CRF-Rs have high affinity for sauvagine, urotensin, and potent CRF antagonists, such as [DPhe<sup>12</sup>, Nle<sup>21,38</sup>]-hCRF(9-41).

#### Example 9

30                   Assay of CRF-RB<sub>1</sub> mediated stimulation

of intracellular cAMP levels

Using methods described in Example 4, the ability of CRF-RB<sub>1</sub> to stimulate cAMP formation in CRF-RB<sub>1</sub>-  
5 expressing COSM6 cells was investigated. The plasmid pCRF-RB<sub>1</sub> was transfected into COSM6 cells. One day later, the cells were trypsinized and replated in 10% FBS, DMEM into 24 or 48 well COSTAR tissue culture wells, and allowed to grow another 24 hours. The medium was changed  
10 to 0.1% FBS, DMEM at least two hours before treatments. The cells were preincubated for 30 minutes with 0.1mM 3-isobutyl-1-methylxanthine and then exposed to various peptides for 30 minutes at 37°C. Intracellular cAMP was measured in duplicate from triplicate wells using an RIA  
15 kit (Biomedical Technologies, Stoughton, MA).

The results are presented in Figure 5. The results indicate that CRF stimulates the accumulation of intracellular cAMP when CRF-RB<sub>1</sub> is transiently transfected  
20 into COSM6 cells. The EC<sub>50</sub> occurs between 1-10 nM, and the dose response is similar to that seen when the mouse analog of CRF-RA<sub>1</sub> is transfected. Urotensin and sauvagine, which are members of the CRF peptide family, are equipotent to CRF in stimulating intracellular cAMP  
25 accumulation. The peptides GRF and VIP do not stimulate cAMP accumulation (see Figure 5). The CRF signal transduced by the cloned CRF-RB<sub>1</sub> receptor is inhibited in the presence of 1 mM CRF antagonist, (DPhe<sup>12</sup>,Nle<sup>21,38</sup>)hCRF(12-41).

Example 10RNase protection assay to determine  
tissue distribution of CRF-RB<sub>1</sub>

5           The coding region for mouse receptor  
corresponding to CRF-RA<sub>1</sub> was cloned by the well-known  
RT-PCR method using primers based on the published  
sequence, and RNA from mouse AtT-20 cells (ATCC No. CCL  
89) as template. Plasmid DNAs encoding amino acids 26-106  
10 of mouse CRF-RA<sub>1</sub> (SEQ ID NO:13) and amino acids 1 to 132  
of mouse CRF-RB<sub>1</sub> (i.e., amino acids 1-132 of SEQ ID NO:9)  
were linearized, and antisense riboprobes were synthesized  
using SP6 RNA polymerase and [ $\alpha$ -<sup>32</sup>P]UTP. An internal  
loading control antisense riboprobe of glyceraldehyde 3-  
15 phosphate dehydrogenase (GAPDH) was synthesized using T7  
RNA polymerase.

RNase protection assays were performed by  
hybridizing 30mg of total RNA from mouse heart and brain  
20 tissues to  $5 \times 10^5$  cpm of labeled riboprobe at 65°C for 18  
hours. This was followed by RNase digestion (180 mg/ml  
RNase A and 350 U/ml RNase T1) at 23°C for 60 minutes,  
after which samples were run on 5% polyacrylamide, 8 M  
urea gels.

25  
RNase protection analysis was used to  
investigate the relative expression of CRF-RA<sub>1</sub> and CRF-RB<sub>1</sub>  
in mouse brain and heart. Whereas both receptors are  
detected in the brain and the heart, the heart appears  
30 primarily to express CRF-RB<sub>1</sub> (which has also been reported



to express CRF mRNA). There are multiple protected fragments seen in the heart. Furthermore, using a 5' CRF-RB<sub>1</sub> probe, the major protected fragment in the brain is smaller than in the heart, and smaller than that expected to be generated from the cloned CRF-RB<sub>1</sub>.

#### Example 11

##### In-situ hybridization assay to determine tissue distribution of CRF-RB<sub>1</sub>

10

The tissue distribution of CRF-RB<sub>1</sub> mRNA was further characterized by in situ hybridization histochemistry using <sup>35</sup>S-labeled antisense cRNA probes. Six week old male C57BL/6 mice were perfused transcardially with 4% paraformaldehyde in 0.1M borate buffer, and regularly spaced series of 20-30 mm thick frozen sections through brain, heart, duodenum and testis/epididymis were taken as described (Simmons et al., J. Histotechnology 12:169-181 (1989)). Radiolabeled antisense and sense (control) cRNA copies were synthesized from a 1.0 kb BamH1 digest of CRF-RB<sub>1</sub> cDNA, encompassing 80 bp of the 5' untranslated region and 926 bp of the CRF-RB<sub>1</sub> coding sequence, subcloned into pBluescript KS vector (Stratagene, La Jolla, CA). <sup>32</sup>S-UTP was used as the radioactive isotope for probe synthesis, and in situ hybridization was performed as previously described (Simmons et al., *supra*; and Imaki et al., Brain Res. 496:35-44 (1989)). Probes were labeled to specific activities of 1-3 X10<sup>9</sup> dpm/mg, and hybridization was

carried out under high stringency conditions (50% formamide with final washes in 0.2 X SSC at 70°C).

Sense-strand cRNAs labeled to similar specific  
5 activities failed to show any suggestion of positive localizations when applied to tissue sections adjoining those in which antisense probes revealed robust signals. Consistent with cloning and RNase protection assay data, CRF-RB<sub>1</sub> transcripts were detected in the heart, where  
10 labeling appeared most prominently over perivascular cells, as well as in the epicardium. In the male reproductive tract, CRF-RB<sub>1</sub> mRNA was localized principally in stromal tissue of the epididymis, while labeling in testis was at or near background levels. CRF-RB<sub>1</sub> mRNA  
15 signal over duodenum appeared as a dense band of silver grains over the submucosal layer, and, additionally, over isolated non-epithelial cells at the base of the villi.

In the brain, CRF-RB<sub>1</sub> mRNA displayed a rather  
20 restricted distribution, which contrasts in extent and topography with that for CRF-RA<sub>1</sub> mRNA in rat. In the septal region, for example, CRF-RB<sub>1</sub> mRNA is expressed in circumscribed aspects of the lateral septal nucleus, while the CRF-RA<sub>1</sub> transcript is seen over the medial septal  
25 complex. Other major sites of CRF-RB<sub>1</sub> mRNA expression in the forebrain include circumscribed aspects of the olfactory bulb, preoptic region, hypothalamus, and amygdala.

In each of these areas, the pattern of CRF-RB<sub>1</sub> expression is seen to be distinct from that of the CRF-RA<sub>1</sub> in rat brain. It appears unlikely that major species differences in CRF-R distribution are at play, since the  
5 mouse CRF-RB<sub>1</sub> probe employed here yielded similar patterns of hybridization in mouse and rat brain.

While the invention has been described in detail with reference to certain preferred embodiments thereof,  
10 it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SUMMARY OF SEQUENCES

Sequence ID No. 1 is the nucleic acid sequence  
(and the deduced amino acid sequence) of a cDNA encoding a  
5 human-derived CRF receptor of the present invention (i.e.,  
hCRF-RA<sub>1</sub>).

Sequence ID No. 2 is the deduced amino acid  
sequence of the human-derived CRF receptor set forth in  
10 Sequence ID No. 1.

Sequence ID No. 3 is the nucleic acid sequence  
(and the deduced amino acid sequence) of a splice variant  
cDNA insert encoding a 29 amino acid insert portion of the  
15 human-derived CRF receptor of the present invention. The  
splice variant cDNA insert is located between nucleotides  
516-517 of Sequence ID No:1 (thereby producing CRF-RA<sub>2</sub>).

Sequence ID No. 4 is the deduced amino acid  
20 sequence of the human-derived CRF receptor splice variant  
insert set forth in Sequence ID No. 3. The splice variant  
amino acid insert is located between amino acids 145-146  
of SEQ ID NO:2.

25 Sequence ID No. 5 is the nucleic acid sequence  
(and the deduced amino acid sequence) of a cDNA encoding  
region of a rat-derived CRF receptor of the present  
invention (i.e., rCRF-RA).

Sequence ID No. 6 is the deduced amino acid sequence of the rat-derived CRF receptor set forth in Sequence ID No. 5.

5               Sequence ID No. 7 is the nucleic acid sequence (and the deduced amino acid sequence) of two exons (less the intervening intron sequence) of a partial genomic clone encoding a mouse-derived CRF receptor of the present invention (i.e., mCRF-RB<sub>1</sub>).

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Sequence ID No. 8 is the deduced amino acid sequence of the human-derived CRF receptor set forth in Sequence ID No. 7.

15               Sequence ID No. 9 is the nucleic acid sequence (and the deduced amino acid sequence) of a cDNA encoding a type-B mouse-derived CRF receptor of the present invention (i.e., mCRF-RB<sub>1</sub>).

20               Sequence ID No. 10 is the deduced amino acid sequence of the mouse-derived CRF-RB receptor set forth in Sequence ID No. 9.

Sequence ID No. 11 is the "sense" probe  
25 described in Example 7.

Sequence ID No. 12 is the "antisense" probe described in Example 7.

Sequence ID No. 13 is the amino acid sequence of the mouse-derived CRF-RA<sub>1</sub> receptor.

Sequence ID No. 14 is the nucleotide sequence of  
5 a splice variant cDNA including an insert encoding a 29 amino acid portion of the human-derived CRF receptor of the present invention. The splice variant cDNA insert is located between nucleotides 516-517 of Sequence ID No:1 (thereby producing CRF-RA<sub>2</sub>).

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Sequence ID No. 15 is the amino sequence of a splice variant CRF (CRF-RA<sub>2</sub>) including a 29 amino acid portion of the human-derived CRF receptor of the present invention. The splice variant amino acid insert is  
15 located between amino acids 145-146 of SEQ ID NO:2.